INTERLEUKIN 12-SECRETING CHIMERIC ANTIGEN RECEPTOR T CELLS AND CANCER IMMUNOTHERAPY

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INTERLEUKIN 12-SECRETING CHIMERIC ANTIGEN RECEPTOR T CELLS AND CANCER IMMUNOTHERAPY

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For an adoptive T cell immunotherapy to successfully treat cancer, T cells must be able to recognize tumor antigen, expand and persist in the tumor microenvironment, and deliver its effector function. Treatment of B cell acute lymphoblastic leukemia (B-ALL) using human T cells modified to express a "second generation" CD19-targeted chimeric antigen receptor (CAR), which includes the CD28 co-stimulatory signaling domain (19-28z) along with the CD3 zeta chain signaling domain, has been successfully demonstrated in both the pre-clinical and clinical settings. However, application of CAR therapy for the treatment of B cell chronic lymphocytic leukemia (B-CLL) and solid tumors remains a challenge. Here, we present preclinical studies demonstrating that further modifying 19-28z CAR T cells to additionally secrete a recombinant fusion protein of the pro-inflammatory interleukin 12 (IL-12) (19-28z/mIL-12 CAR T cells) enhances the potency of adoptive T cell immunotherapy. We demonstrate that these 19-28z/mIL-12 "armored" CAR T cells display increased proliferation, up-regulation of

perforin and granzyme B, as well as a central memory-like phenotype when compared to T cells transduced to express the 19-28z CAR alone. *In vivo*, treatment of systemic NALM-6 B-ALL tumor-bearing SCID-Beige mice with 19-28z/mIL-12 CAR T cells significantly improved survival when compared to mice treated with T cells modified to express the 19-28z CAR alone. Furthermore, in contrast to T cells modified to express the 19-28z CAR alone, 19-28z/mIL-12 CAR T cells resist $CD4^{+}CD25^{hi}FOXP3^{+}$ Treg-mediated immunosuppression *in vitro* and successfully eradicate Raji Burkitt's lymphoma tumor in SCID-Beige tumor-bearing mice previously infused with CD19-targeted CAR modified Tregs. These findings have important implications for future clinical trials with CAR T cells designed to enhance the clinical outcomes of relapsed B-CLL as well as solid tumor malignancies.

BIOGRAPHICAL SKETCH

Peter Sho-Yao Chang was born and raised in Honolulu, Hawaii. After graduating from Kaimuki High School as a valedictorian of his class in 2002, he attended the University of Hawaii at Manoa (UHM) from 2002 to 2006, majoring in biology and minoring in chemistry. In 2005, Peter joined the Haumana Research Training Program, a research program provided by the Pacific Biosciences Research Center at UHM for training undergraduate science majors toward a future in Ph.D. graduate research. Under the guidance of Dr. Marla J. Berry, Peter worked on a study examining the role of selenium and selenoprotein in allergic airway inflammation which was published in the *Journal of Immunology* in 2007. After receiving his Bachelor of Science degree in 2006, Peter enrolled in the University of Pennsylvania Post-Baccalaureate Research Education Program (PennPREP) to further his research training under the guidance of Dr. Susan R. Ross. In 2008, Peter began his Ph.D. training in the Immunology and Microbial Pathogenesis Program at Weill Cornell Graduate School of Medical Sciences of Cornell University. He joined Dr. Renier J. Brentjens' laboratory at Memorial Sloan Kettering Cancer Center in December 2010 and finished his Ph.D. research, investigating IL-12-secreting chimeric antigen receptor-modified T cells as a potential therapy for cancer.

This dissertation is dedicated to my beloved parents Nancy Shu Hui and Pin-Pu Chang. Mom, my achievements would not be possible without your unconditional love and support over the years. Dad, your passing a decade and a half ago has been an inspiration for my pursuit in cancer research.

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LIST OF ABBREVIATIONS

AAPC	artificial antigen presenting cell
ACT	adoptive cell transfer
AHR	airway hyperresponsiveness
AICD	activation-induced cell death
ALL	acute lymphoblastic leukemia
APC	antigen presenting cell
BMT	bone marrow transplantation
CAR	chimeric antigen receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CR	complete remission
DLI	donor lymphocyte infusion
DCS	donor calf serum
DLBCL	diffuse large B cell lymphoma

DMEM	Dulbecco’s Modified Eagle Medium
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
FL	follicular lymphoma
GaLV	gibbon ape leukemia virus
GFP	green fluorescent protein
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
GzmB	granzyme B
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
IRES	internal ribosome entry site
i.t.	intratumoral

i.v.	intravenous
IVIG	intravenous immunoglobulin
MDSC	myeloid-derived suppressor cell
MRD	minimal residual disease
MZL	marginal zone lymphoma
NALM	null acute leukemia-Minowada
NHL	non-Hodgkin’s B cell lymphoma
NK	natural killer cell
NKSF	natural killer stimulatory factor
NKT	natural killer T cell
NOD	non-obese diabetic
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed death 1
PD-L1	programmed death-ligand 1
PE	phycoerythrin
Pfn	perforin

PHA	phytohemagglutinin
PR	partial remission
PSMA	prostate-specific membrane antigen
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
scFv	signal chain variable fragment
SCID	severe combined immunodeficiency
SD	stable disease
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TAM	tumor-associated macrophage
T_{CM}	central memory T cell
TCR	T cell receptor
Teff	effector T cell
T_{EM}	effector memory T cell

TGF-β	tumor growth factor beta
Th	T helper cell
TIL	tumor infiltrating lymphocyte
TLR	toll-like receptor
Treg	regulatory T cell
T_{SCM}	stem cell memory T cell
WT1	Wilms' tumor gene 1

CHAPTER 1: INTRODUCTION

Cancer Immunotherapy

As early as the 1700s, evidence of spontaneous remission of malignant disease in cancer patients with concomitant bacterial infection has been documented (Starnes, 1992). However, it was not until the 1890s when William Coley, a New York surgeon, first took advantage of this phenomenon in benefiting cancer patients. Coley observed that sarcoma patients who suffered from erysipelas, an acute *Streptococcus* bacterial infection, had spontaneous tumor regression (Coley, 1896). Attributing the tumor regression to toxins induced by the *Streptococcus* bacteria, Coley initially treated sarcoma patients with live *Streptococcus* infection. However, the risk of uncontrollable *Streptococcus* infection led to the use of a mixture composed of heat-killed Gram-positive *Streptococcus pyrogenes* combined with Gram-negative *Serratia marcescens*. Coley's toxin, as such mixture of Gram-positive and negative bacteria has come to be known, has since been applied in many types of cancer with inconsistent results. While the exact mechanism of action in Coley's toxin was not known at the time, the early success of this therapy has opened the field of cancer immunotherapy (Starnes, 1992).

The principle that T cells can eradicate tumor can be traced to evidence of graft-versus-leukemia (GVL) in patients undergoing allogeneic bone marrow transplantation (allo-BMT) for hematological malignancies. Patients undergoing allo-BMT have lower risk of relapse when compared to receiving syngeneic BMT. Graft-versus-host disease (GVHD) following BMT is also associated with reduced risk of relapse. The lower risk

of relapse as seen in allo-BMT and GVHD can be attributed to GVL of T cells attacking host tumor as T cell depletion resulted in increased risk of relapse (Porter and Antin, 1999). Moreover, adoptive T cell therapy utilizing donor-derived T cells, a procedure known as donor lymphocyte infusion (DLI), induces tumor remission in relapsed patients of chronic myelogenous leukemia (CML) following allo-BMT (Cullis et al., 1992).

The presence of lymphocyte infiltration in spontaneous cancer regression provides further evidence of T cell-mediated immunity against tumor (Rosenberg et al., 1972). Human tumor infiltrating lymphocytes (TILs) derived from resected melanoma contain T cells that specifically recognize autologous tumor (Muul et al., 1987). This knowledge led to the first demonstration that adoptively transferring autologous TILs can mediate tumor regression in metastatic melanoma (Rosenberg et al., 1988). Adoptive cell transfer (ACT) therapy with TIL infusion has thus far achieved success in melanoma and cervical cancer (Rosenberg and Restifo, 2015). In addition to TILs, host T cells can be genetically modified to express anti-tumor T cell receptors (TCRs) and chimeric antigen receptors (CARs) for ACT. ACT therapy with TCR-modified T cells targeting the NY-ESO-1 antigen expressed in synovial sarcoma was the first reported targeting of nonmelanoma solid tumor (Robbins et al., 2011). Recently, ACT therapy with CAR-modified T cells also showed promising results in the treatment of hematological malignancies (van der Stegen et al., 2015).

Chimeric Antigen Receptor T Cells

The history of chimeric antigen receptors (CARs) can be traced back to the early 1990s after the ζ-chain of the CD3 complex was first cloned (Weissman et al., 1988). Pivotal studies on leukemic T cells demonstrated the feasibility of inducing activation of T cells expressing chimeric receptors composed of CD3 ζ-chain fused to the extracellular domains of CD8, CD4, or CD25 (Irving and Weiss, 1991; Letourneur and Klausner, 1991; Romeo and Seed, 1991). Eshhar and colleagues further generated the first CAR by fusing a single-chain variable fragment (scFv) to the CD3 ζ-chain (Eshhar et al., 1993). This scFv, derived from the variable fragments of the immunoglobulin heavy and light chains, can induce T cell activation upon recognition of the hapten trinitrophenyl. The success in Eshhar's seminal study on scFv-CD3ζ CAR meant that it was possible to induce T cell activation in response to virtually any surface-exposed antigen to which a cognate scFv can be generated. This is an attractive tool that can be applied to cancer immunological research as T cells can be engineered to express a CAR that target any surface-exposed tumor associated antigen (TAA).

T Cell Activation and CAR Design: In the classical definition of T cell activation, a T cell requires two signals to be fully activated. The first signal (signal 1) is provided by antigen-specific recognition of TCR (Smith-Garvin et al., 2009). TCR signaling can further be amplified and modulated by co-stimulation (signal 2) provided by antigen presenting cells (APCs). A classic example of co-stimulation is CD80 and CD86 expressed on the surface of APCs that stimulate CD28 expressed on the surface of T cells. Co-stimulatory receptors include both constitutively expressed moieties such

as CD28 and CD27 as well as activation-induced moieties such as 4-1BB, GITR, OX-40, and ICOS (Sanmamed et al., 2015). TCR engagement in the absence of co-stimulation may lead to a T cell unresponsive state known as anergy. Co-stimulation protects T cells from anergy and activation-induced cell death (AICD). Once a T cell becomes fully activated, the T cells rapidly proliferate and upregulate cytotoxic molecules. A proportion of the activated T cells eventually differentiate into memory T cells. These long-lived T cells can be loosely divided into two major types: effector memory (T_{EM}) and central memory (T_{CM}) T cells. T_{EM} cells are more differentiated as characterized by reduction of CCR7 and CD62L expression. T_{CM} cells are less differentiated and retain high expression of CCR7 and CD62L (Kaech and Cui, 2012).

CAR Development

A chimeric antigen receptor (CAR) is a fusion receptor that combines antigen recognition property of an immunoglobulin with T cell activation. A T cell engineered to express CAR, therefore, acquires the ability to become activated upon recognizing cell surface antigens in a manner analogous to an antibody. This unique property of CAR T cells has recently emerged to become a promising area of research in the field of cancer immunotherapy.

First Generation CAR

Eshhar's TNP-targeted CAR is now considered to be a first generation CAR (Figure 1.1). A first generation CAR provides only one signal (signal 1) to a CAR-expressing T cell via CD3ζ induction of downstream phosphatidyl inositol and tyrosine kinase pathways along with calcium influx, which then leads to cytotoxic activity

against the target cell (Sadelain et al., 2013). Gong and colleagues first demonstrated the efficacy of first generation CAR in the context of tumor cells by generating a chimeric antigen receptor specific to prostate-specific membrane antigen (PSMA) known as Pz1 (Gong et al., 1999). Pz1 CAR T cells were demonstrated to readily lyse prostate cancer cells *in vitro*. However, Pz1 CAR T cells did not readily secrete IL-2 and were unable to expand upon repeated antigen exposure. 19z1 CAR T cells, which targets the common B cell antigen CD19 expressed on B cell tumors, show similar anti-tumor cytotoxic activity *in vitro*, but have limited anti-tumor response *in vivo* (Brentjens et al., 2003). It became apparent that co-stimulation is necessary to successfully induce a sustained CAR T cell anti-tumor response *in vivo*. Brentjens and colleagues demonstrated that 19z1 CAR T cells can eradicate tumor cells that express the co-stimulatory molecule CD80 such as Raji Burkitt's lymphoma tumor cells *in vivo,* but have limited anti-tumor efficacy against CD80 deficient acute lymphoblastic leukemia (ALL) NALM-6 tumor cell line.

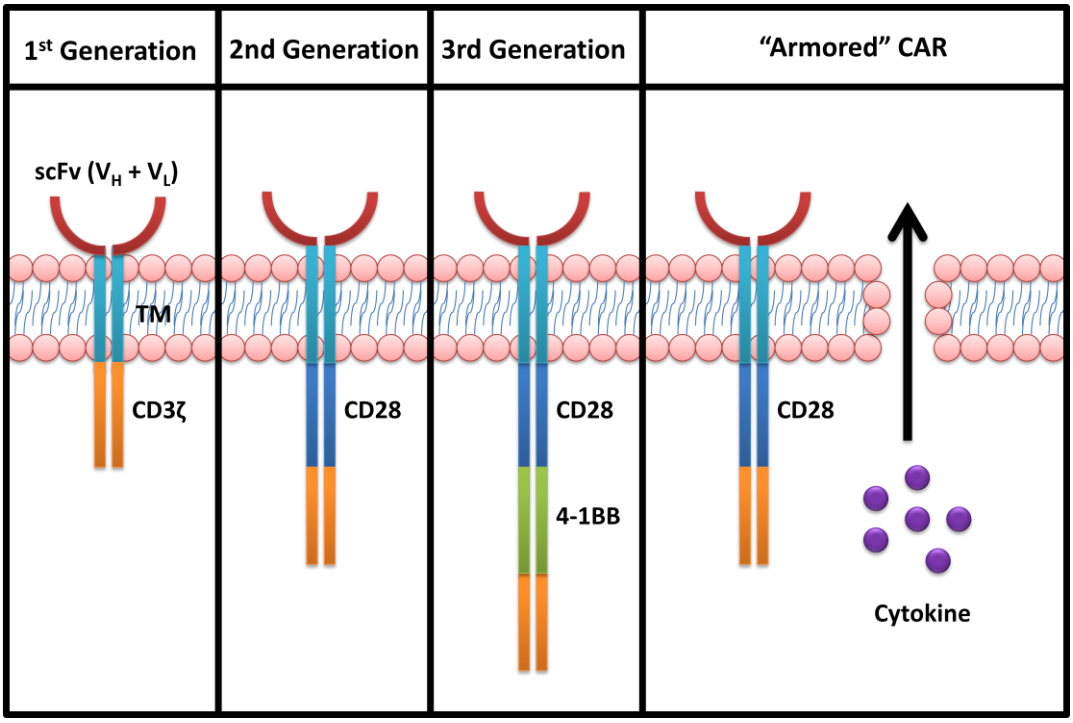

Figure 1.1 Development of chimeric antigen receptors
First generation CARs contain the cytoplasmic signaling domain of the CD3 TCRζ chain. *Second generation* CARs contain the cytoplasmic signaling domain of the TCRζ chain and one additional cytoplasmic signalling domain of co-stimulatory receptors such as CD28, 4-1BB, and OX-40. *Third generation* CARs contain two signalling domains of costimulatory receptors in addition to the signalling domain of CD3 TCRζ chain. "*Armored" CAR* introduces additional genes such as cytokines or co-stimulatory ligands to a CAR to enhance the function of CAR T cells. *TM, transmembrane domain*.

Second Generation CAR

While preliminary data suggested the need for CAR T cells to receive co-stimulation, many tumors fail to express co-stimulatory ligands. The limited success of first generation CAR T cells *in vivo* led to the generation of CAR T cells that incorporate the signaling domain of co-stimulatory molecules, now collectively known as second

generation CAR T cells (Figure 1.1). This provides signal 2 in addition to signal 1, preventing T cell anergy. Brentjens and colleagues at Memorial Sloan Kettering Cancer Center (MSKCC) tested whether incorporating the co-stimulatory molecules CD28, DAP10, 4-1BB, and OX-40 in the CAR design enhances the anti-tumor efficacy of CAR T cells (Brentjens et al., 2007). Among these, 19-28z CAR T cells, which incorporates the signaling domain of CD28 in the CAR, was demonstrated to have the most robust phenotype as characterized by enhanced proliferation and increased secretion of IL-2, a marker of T cell co-stimulation, as well as IFN-γ *in vitro* in comparison to the other co-stimulatory CAR receptors tested. More importantly, 19-28z CAR T cell therapy enhances the survival of NALM-6 tumor-bearing SCID-Beige mice as compared to mice treated with 19z1 CAR T cells (Brentjens et al., 2007). Rosenberg and colleagues at the National Cancer Institute (NCI) further confirmed these findings by comparing a similar CD19-targeted CAR that incorporate the CD28 and 4-1BB signaling domains. The CD19-targeted CAR T cells lysed primary human chronic lymphocytic leukemia cells (CLL) in which the CD28 signaling domain-containing CAR T cells produce more IFN-γ and IL-2 as compared to the 4-1BB CAR *in vitro* (Kochenderfer et al., 2010). June and colleagues at the University of Pennsylvania (UPenn) confirmed the superior anti-tumor response of second generation CAR T cells compared to first generation CAR T cells, but 4-1BB CAR T cells were shown to survive longer in the context of a xenotransplant tumor mouse model as compared to CD28 CAR T cells (Milone et al., 2009). Investigators at the Fred Hutchinson Cancer Center (Fred Hutch), Baylor College of Medicine (Baylor), and MD Anderson have also generated versions of second generation CAR incorporating either a CD28 or 4-1BB signaling moiety in their designs,

demonstrating anti-tumor efficacy in the context of B cell tumors (Kowolik et al., 2006; Terakura et al., 2012; Vera et al., 2006).

The design of the second generation CAR T cells at these institutions differ in many respects. The key differences among the six different institutions are summarized in Table 1.1. Besides differences in the use of CD28 or 4-1BB as co-stimulatory molecules, the spacer regions between the scFv and co-stimulatory signaling domain as well as the method of genetic modification also differ among the groups. The majority of institutions utilizes a viral vector, either retrovirus or lentivirus, for genetic modification. On the other hand, Cooper and colleagues have developed the *Sleeping Beauty System* that integrates DNA into the genome by means of electroporation of DNA plasmids carrying transposon and transposase (Singh et al., 2008). The transposon carrying the CAR can efficiently integrate at random sites in the genome (Morgan and Kakarla, 2011). It remains to be determined whether CD28 or 4-1BB containing second generation CAR T cells has superior anti-tumor efficacy. Collectively, the early pre-clinical studies have demonstrated superior anti-tumor efficacy of second generation CAR T cells as compared to first generation CAR T cells. These studies have led to early success in clinical trials for the treatment of some hematological tumors such as B cell acute lymphoblastic leukemia (B-ALL).

Due to lack of *in vivo* efficacy of first generation CAR T cells for the treatment of B cell tumors in pre-clinical studies, most of the early clinical trials have been largely focused on second generation CAR T cells with the exception of Brenner and colleagues, who treated patients in a clinical trial with a combinational therapy of CD19-targeted

CAR T cells that encodes the CD3ζ domain alone or both CD28 and CD3ζ domains (Savoldo et al., 2011). The injected CAR T cells were monitored and the results demonstrated enhanced persistence of the CAR T cells expressing the CD28 signaling domain as compared to the CAR T cells lacking a CD28 signaling domain.

Table 1.1 CD19-targeted chimeric antigen receptor design

Institute	scFv clone	Spacer regions	Signaling Domains	Genetic modification methods
Baylor	FMC63	Human IgG1 CH_2CH_3 domain	CD28 costimulation CD3ζ activation	Retroviral transduction
NCI	FMC63	No hinge	CD28 costimulation CD3ζ activation	Retroviral transduction
MD Anderson	FMC63	Modified human IgG_4	CD28 costimulation CD3ζ activation	Sleeping beauty transposon Electroporation
MSKCC	SJ25C1	No hinge	CD28 costimulation CD3ζ activation	Retroviral transduction
UPenn	FMC63	Human CD8α hinge	4-1BB costimulation CD3ζ activation	Lentiviral transduction
Fred Hutch	FMC63	Modified human IgG	4-1BB costimulation CD3ζ activation	Lentiviral transduction

Third Generation CAR

In addition to first and second generation CAR T cells, many have investigated combining two co-stimulatory domains within the cytoplasmic tail in CAR design (Zhong et al., 2010). However, these so-called third generation CAR T cells do not necessarily improve the efficacy of CAR therapy as compared to therapy with second generation CAR T cells (Morgan et al., 2010). For example, Tamma and colleagues

engineered CD19-targeted CAR containing an intracellular domain of CD3ζ chain along with both 4-1BB and CD28 domains (1928BBζ). Treatment of 1928BBζ CAR T cell therapy resulted in delayed tumor progression and enhanced survival of NOD/SCID CD19-tumor bearing mice compared to second generation CAR treatment (Tammana et al., 2010). This improved therapeutic benefit was also seen in a PSMA-targeted third generation CAR T cells expressing both CD28ζ and 4-1BB domains (Zhong et al., 2010). On the other hand, no therapeutic benefit was observed in third generation CD28ζ and 4-1BB domain-expressing CAR T cells engineered by Milone and colleagues (Milone et al., 2009).

Advantages of CAR T cells

CAR T cells have a number of distinct advantages compared to T cells engineered to express tumor-specific TCRs. It is well known that cancer cells downregulate human leukocyte antigen (HLA) as a mechanism of immune escape (Hicklin et al., 1999). While a TCR can only recognize protein antigen in the context of HLA, a CAR can recognize any surface antigen independent of HLA expression. In addition to proteins, a CAR can also recognize carbohydrates and glycolipids, expanding potential targets (Sadelain et al., 2013). Until now, CAR T cells were known to target only native cell surface antigens, limiting the repertoire of TAAs. However, a preliminary study demonstrating the feasibility of CAR T cells to target the human intracellular protein, Wilms' tumor gene 1 (WT1) in the context of HLA-A0201 has been reported at a recent American Society of Hematology (ASH) annual meeting (Rafiq et al., 2014).

Clinical Studies with CD19-Targeted CAR T Cells

B-cell hematological malignancies have been extensively studied in clinical trials using second generation CAR T cells. CD19, a relatively ideal tumor associated antigen (TAA), has been the main target used to treat most B cell malignancies. CD19 is expressed in most B-cell leukemias and lymphomas and its expression in normal tissues is limited to the B cell lineage. While targeting CD19 results in B cell aplasia, such "on-target off-tumor" toxicity can be managed with infusions of intravenous immunoglobulin (IVIG) (Anderson et al., 2007).

B-ALL: Among the B cell malignancies investigated in clinical trials, treating B cell acute lymphoblastic leukemia (B-ALL) with second generation CD19 CAR therapy has demonstrated the most promising clinical results. B-ALL is an acute form of leukemia that is characterized by the accumulation of malignant pre-B cell lymphoblasts. Until the advent of CAR therapy, patients with B-ALL had few treatment options, largely a combination of chemotherapy to induce remission followed by consolidation and maintenance of residual disease (Bassan and Hoelzer, 2011).

While complete remission (CR) has reached about 85-90% over the last decade using conventional methods for the treatment of B-ALL among pediatric patients, it remains a challenge to treat the adult form of B-ALL. Until the advent of CAR therapy, salvage therapy for relapsed B-ALL has a historical remission rate of about 30% (Gökbuget et al., 2012). Brentjens and colleagues at MSKCC were the first to report the results of CD19 CAR T cell therapy trial for the treatment of relapsed B-ALL. Among the 16 patients treated with CD19 CAR T cell therapy, there was an 88% overall rate of

patients with CR in which 44% of the patients received "standard-of-care" allogeneic hematopoietic stem cell transplantation (HSCT) (Brentjens et al., 2013; Davila et al., 2014). Grupp and colleagues at UPenn reported 90% CR among the 30 patients treated in their clinical trial (Maude et al., 2014). Rosenberg and colleagues at NCI reported an overall CR rate of 70% among the 21 pediatric and young adult patients that were treated with CD19 CAR therapy. Ten of the 12 patients with minimal residual disease (MRD)-negative status received HSCT (Lee et al., 2015). Early clinical results from MD Anderson and Fred Hutchinson Center have also been reported at a recent ASH annual meeting with some encouraging results (Kebriaei et al., 2014; Turtle et al., 2014).

B-CLL: B-CLL is the most common form of hematologic malignancy in the Western world, and it is characterized by the accumulation of long-lived B lymphocytes in the bone marrow, lymphoid organs, and peripheral blood. B-CLLs are inefficient antigen presenting cells (APCs) with low expression of T cell co-stimulatory receptors CD80 and CD86, but expression of these co-stimulatory ligands can be induced by CD40L and soluble factors secreted by activated T cells (Romano et al., 2003).

In contrast to the clinical trial results for the treatment of B-ALL using second generation CD19 CAR treatment, results in low grade B cell malignancies such as B-CLL have been far more modest (Brentjens et al., 2011; Kochenderfer et al., 2013). June and colleagues at UPenn initially reported minimal residual disease MRD^- CR in 2 of 3 B-CLL patients treated with CD19 CAR therapy (Kalos et al., 2011; Porter et al., 2011). However, a more recent report on the result of 14 patients with relapsed and refractory B-CLL showed an overall response rate of 57% (8/14) of which four of the patients had

CR and four others had partial remission (PR) (Porter et al., 2015). In the study conducted at MSKCC, seven B-CLL patients were treated with CAR T cell therapy, four of which received preconditioning chemotherapy prior to treatment. None of the patients who received chemotherapy had a response, while three of the four patients who received chemotherapy experienced stable disease following CAR T cell infusion (Brentjens et al., 2011). Clinical studies reported at NCI described four patients who were treated with CAR T cell infusion after preconditioning with cyclophosphamide and fludarabine. Three of the four patients achieved classically defined CRs (Kochenderfer et al., 2015, 2012).

DLBCL: In addition to B-ALL and B-CLL, clinical results have also been reported by multiple centers testing the efficacy of CD19 CAR therapy for the treatment of diffuse large B cell lymphoma (DLBCL). While 50-80% of patients with DLBCL are cured with standard induction therapy, relapse is the major cause of treatment failure that ultimately results in mortality of patients. MSKCC has reported preliminary results of 100% CR among the two follicular lymphoma (FL), three relapsed DLBCL, and one marginal zone lymphoma (MZL) patients treated with CAR T cell therapy (Sauter et al., 2014). Kochenderfer and colleagues at NCI reported 1 CR and 4 PR among the eight DLBCL patients treated with CAR T cell therapy (Kochenderfer et al., 2014). Preliminary finding from a clinical trial at UPenn resulted in 2 CR among the 6 DLBCL patients treated with CAR therapy (Schuster et al., 2014). At MD Anderson, among the five DLBCL or FL patients treated, four patients achieved CR and one patient achieved PR (Kebriaei et al., 2014).

While these early clinical trials show great promise in the treatment of B-ALL with CD19 CAR therapy, several challenges of this therapy remains to be explored. First, all of the clinical trials thus far require the administration of a lymphodepletion regimen such as cyclophosphamide and fludarabine treatment prior to CAR T cell infusion. This can pose a challenge for elderly immunocompromised patients who may not be able to tolerate such a toxic treatment. CAR T cell therapy also induces neurological abnormalities characterized by seizures, aphasia, and encephalopathy. Furthermore, one of the major side effects of CAR therapy is the induction of large quantity of inflammatory cytokines that, on the one hand, promotes CAR T cell function and, on the other hand, also induces fever, hypotension, and hypoxia. These symptoms associated with increase in serum inflammatory cytokines are collectively termed cytokine release syndrome (CRS). C-reactive protein (CRP) have recently been identified as an indicator measuring the severity of CRS, and IL-6R blockade (tocilizumab) have been demonstrated to protect patients from the toxicities associated with CRS (Grupp et al., 2013).

Etiologies of CAR T cell Treatment Failure

Despite the successful clinical studies in treating patients with B-ALL, treating B-CLL and solid tumors remains a challenge. This can possibly be attributed to the immunosuppressive tumor microenvironment of B-CLL and solid tumors. Accumulation of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor associated macrophages (TAMs) as well as the secretion of immunosuppressive cytokines and angiogenic factors all play a critical role in promoting tumor progression

and suppressing the function of CD4 and CD8 effector T cells (Teff) in the tumor microenvironment.

Regulatory T cells (Tregs): Tregs, a specific subset of T cells that function to maintain immune tolerance, can be broadly classified into two groups. Natural Tregs (nTregs) are $CD4^{+}CD25^{+}CD127^{lo}FOXP3^{+}$ T cells derived from the thymus that comprise approximately 5-10% of the CD4 T cell population in human and mice. Naïve CD4 T cells can also be induced with TGF-β and IL-2 to acquire suppressive function. $CD4^{+}CD25^{+}FOXP3^{+}$ T cells derived from Teff cells in the periphery are known as induced Tregs (iTregs). Tregs were initially thought to function in a contact-dependent manner when Sakaguchi and colleagues demonstrated that adding neutralizing antibodies against TGF-β and IL-10 have no effect on Treg suppression *in vitro* (Takahashi et al., 1998). In contrast, more recent studies on the role of Tregs in controlling airway allergic responses demonstrated the possible role of IL-10 and TGF-β in the suppressive function of Tregs *in vivo* (Hawrylowicz and O'Garra, 2005). Joetham and colleagues demonstrated that injection of IL-10 deficient Tregs had no effect on reducing airway hyperresponsiveness (AHR) of allergen challenged mice, whereas pre-incubation of Tregs with IL-10 before Treg transfer resulted in AHR suppression associated with elevated levels of bronchoalveolar lavage TGF-β levels (Joetham et al., 2007). Moreover, Vignali and colleagues reported that Tregs can suppress in a contact independent mechanism mediated by IL-35 secretion (Chaturvedi et al., 2011; Collison et al., 2007). By virtue of expressing the high affinity IL-2 receptor CD25, Tregs have also been proposed to function as a "cytokine sink" that deprives

actively dividing Teff cells of IL-2 essential for their survival (Thornton and Shevach, 1998).

The association of poor prognosis and Treg accumulation in human cancer has been well-documented. Zou and colleagues identified preferential accumulation of Tregs in advanced staged ovarian cancer patients (Curiel et al., 2004). $FOXP3^+$ Tregs accumulate in high number among invasive breast cancer patients and has been identified as a risk factor of breast cancer relapse (Bates et al., 2006). In a correlation study of lung cancer patients, Patz and colleagues demonstrated that high infiltrating Treg/TIL ratio is associated with high risk of lung cancer relapse (Petersen et al., 2006). Others have also identified the negative risk factor of high Treg frequency in the context of hepatocellular carcinoma (Gao et al., 2007), renal cell carcinoma (Griffiths et al., 2007), gastric cancer (Perrone et al., 2008), cervical cancer (Jordanova et al., 2008), and colorectal carcinoma (Sinicrope et al., 2009). Other than solid tumors, extensive studies have confirmed the increased levels of Tregs in the context of B-CLL, especially among patients at advanced stage of the disease (Jadidi-Niaragh et al., 2013).

Myeloid-Derived Suppressor Cells (MDSCs): MDSCs are $CD11b^+GR1^+$ immature cells that exhibit strong immunosuppressive function in the context of cancer (Gabrilovich et al., 2012). The immunosuppressive effect of MDSCs has been examined in many types of cancer, including melanoma (Mandruzzato et al., 2009), colon cancer (Mandruzzato et al., 2009), prostate cancer (Vuk-Pavlović et al., 2010), and hepatocellular carcinoma (Hoechst et al., 2008). MDSC can broadly be divided into two types: monocytic MDSC and polymorphonuclear MDSC. Monocytic MDSCs express

monocyte markers F4/80, CD115, and CCR2, and suppress CD8 T cells predominantly by depriving CD8 T cells of L-ariginine through ARG1 expression and increasing the production of reactive nitrogen species (RNS) (Gabrilovich et al., 2012). Polymorphonuclear MDSCs are morphologically similar to neutrophils, but are less phagocytic. High levels of ARG1 expression and increased reactive oxygen species (ROS) enable polymorphonuclear MDSCs to suppress activated T cells (Gabrilovich et al., 2012).

Tumor Associated Macrophages (TAMs): In addition to MDSCs, macrophages that accumulate in the tumor microenvironment can acquire an immunosuppressive phenotype. These tumor-associated macrophages (TAMs) exhibit an M2 phenotype characterized by the high levels of IL-10 and low levels of IL-12 expression. In contrast, "classically activated" M1 macrophages are characterized by high levels of IL-12 and low levels of IL-10 expression and are tumoricidal (Gabrilovich et al., 2012). TAMs can suppress immunity by several mechanisms. Secretion of CCL22 by TAMs recruits Tregs to the tumor, and secretion of TGF-β and prostaglandin E2 (PGE2) further contribute to immune suppression (Curiel et al., 2004; Gabrilovich et al., 2012). TAMs can also directly induce apoptosis of PD-1^+ T cells via PD-L1 expression (Kuang et al., 2009).

Programmed death ligand 1 (PD-L1): PD-L1, also known as B7-H1, belongs to the B7 family of ligands that include co-stimulatory molecules such as CD80 and ICOS-L (Collins et al., 2005). PD-L1 is overexpressed in both murine and human tumors (Keir et al., 2008). Programmed death 1 (PD-1), the receptor of PD-L1, is

expressed on the surface of activated T cells. PD-L1 ligation inhibits CD3/CD28-induced Akt activity which in turn leads to reduction in the expression of BCL-XL, an antiapoptotic cell survival factor (Parry et al., 2005). This also results in decreased proliferation and IL-2 production (Carter et al., 2002). Overcoming PD-L1-mediated immune suppression is therefore another critical aspect of the tumor microenvironment.

Interleukin 12 and CAR T Cell Therapy

IL-12 Structure: Interleukin 12 (IL-12) is a heterodimeric protein composed of two subunits with molecular mass of 35 (IL-12-p35) and 40 (IL-12-p40) kDs. Covalently linked IL-12-p35 and p40 subunits combine to form IL-12-p70, a cytokine that has pleiotropic effect in many cell types of the immune system (Lasek et al., 2014). IL-12-p40 can also form homodimers of IL-12-p40/p40 that suppress the biological effect of IL-12-p70 via receptor competition (Ling et al., 1995; Mattner et al., 1993). For the purpose of this thesis, IL-12 will refer to the heterodimer IL-12-p70 form.

IL-12 receptor: The receptor of IL-12 is composed of two amino acid chains, IL-12Rβ1 and IL-12Rβ2. IL-12Rβ1 is constitutively expressed in peripheral blood mononuclear cells (PBMCs) and widely expressed in most cell lines in the hematopoietic lineage but absent in the nonhematopoeitic lineage (Wu et al., 1996). IL-12Rβ2 is not expressed in naïve T cells, but induced upon T cell receptor activation. IL-12, IFN-γ, TNF-α, and CD28 stimulation can further augment IL-12Rβ2 expression in activated T cells (Afkarian et al., 2002; Rogge et al., 1997; Szabo et al., 1997). IL-12Rβ2 expression has also been observed in natural killer (NK) cells, natural killer T (NKT) cells, myeloid cells and tonsillar B cells (Airoldi et al., 2000; Grohmann et al., 1998;

Presky et al., 1996). IL-12Rβ1 is primarily responsible for IL-12 binding, while IL-12Rβ2 is required for IL-12 signaling (Presky et al., 1996). IL-12 induces Janus kinase 2 (Jak2) and tyrosine kinase 2 (Tyk2) phosphorylation, which in turn leads to signal transducer and activator of transcription (STAT)-4 phosphorylation in T and NK cells (Bacon et al., 1995; Thierfelder et al., 1996).

IL-12 function: IL-12 was originally discovered in 1989 and it was known as Natural Killer cell stimulatory factor (NKSF) due to its biological effect on NK cells (Kobayashi et al., 1989). IL-12 is primarily produced by antigen presenting cells (APCs) such as macrophages, dendritic cells, and B cells upon Toll-like receptor (TLR) engagement (Tugues et al., 2015). IFN-γ, IL-15, and CD40L stimulation on APCs can further amplify the production of biologically active IL-12 (Kuwajima et al., 2006; Ma et al., 1996; Schulz et al., 2000). On the other hand, TGF-β1 and IL-10 can negatively regulate IL-12 production (D'Andrea et al., 1993; Du and Sriram, 1998). IL-12 has been shown to enhance the proliferation of NK cells and T cells (Perussia et al., 1992). Furthermore, IL-12 has the potential to enhance cytotoxicity in NK cells and T cells by upregulating granzyme B and perforin (Aste-Amezaga et al., 1994; Sakedo et al., 1993). IL-12 also functions to promote Th1 immune response by inducing IFN-γ production while inhibiting the differentiation of Th2, Treg, and Th17 cells (Djuretic et al., 2007; Manetti et al., 1993; Prochazkova et al., 2012). Enhanced IFN-γ secretion further leads to enhanced IgG2a B cell class-switching, while IL-12 inhibits IgE production (Morris et al., 1994).

IL-12-mediated tumor suppression: The therapeutic benefit of IL-12 as an agent to induce anti-tumor response has been recognized as early as 1993 when Brunda and colleagues reported intraperitoneal (i.p.) administration of IL-12 induced antitumor response against B16 melanoma, M5975 sarcoma, and RENCA renal cell carcinoma (Brunda et al., 1993). This anti-tumor response was in part dependent on CD8 T cells as depletion of CD8 T cells resulted in decreased anti-tumor efficacy in the study. The contribution of NK and NKT cells in IL-12-mediated anti-tumor response has also been investigated in EL4-S3 lymphoma, B16F10 melanoma, and RM-1 prostate carcinoma (Smyth et al., 2000). Interestingly, it was demonstrated that NKT cells played a critical role in anti-tumor efficacy at low dose and delayed IL-12 treatment, while NK cells were the primary catalyst under high dose IL-12 treatment conditions. Since Brunda's early studies, preclinical studies on systemic intravenous (i.v.), intraperitoneal (i.p.), subcutaneous (s.c.), and intratumoral (i.t.) delivery of recombinant IL-12 have all indicated promising therapeutic potential of IL-12 against various immune mouse models of cancer (Colombo and Trinchieri, 2002).

Despite the promising results in preclinical studies of IL-12-mediated suppression, high toxicity of systemic recombinant IL-12 administration presents a major impediment for clinical application (Leonard et al., 1997). Furthermore, early clinical studies in metastatic renal carcinoma, melanoma, colon carcinoma, recurrent ovarian cancer, and neck and head carcinoma showed very few PRs and CRs among i.v. IL-12-treated patients (Atkins et al., 1997; Hurteau et al., 2001; Leonard et al., 1997). In contrast to systemic administration of IL-12, localized i.t. delivery of a plasmid encoding IL-12 showed some clinical benefits in melanoma (Heinzerling et al., 2005;

Mahvi et al., 2007). Moreover, encouraging clinical results have been demonstrated in s.c. administration of IL-12 in hematological malignancies. For instance, an overall response rate of 56% was seen in cutaneous T cell lymphoma patients receiving s.c. IL-12 treatment (Rook et al., 1999). S.c. IL-12 treatment of non-Hodgkin's B cell lymphoma (NHL) patients resulted in 21% PR or CR and about 50% stable disease (SD) (Younes et al., 2004). Treating NHL with s.c. IL-12 in combination with anti-CD20 rituximab further improved clinical outcomes, resulting in 25% CR and 42% PR (Ansell et al., 2002).

The therapeutic potential of IL-12 in tumor suppression and the promising results of second generation CAR T cells in cancer immunotherapy prompted the studies in this dissertation. Combining IL-12 and CAR T cells in the form of an IL-12-secreting CAR T cells may improve CAR T cell therapy and possibly overcome the suppressive tumor microenvironment. In this dissertation, we describe the effect of IL-12 on the biology of CAR T cells and demonstrate its therapeutic potential in enhancing anti-tumor efficacy of CAR T cell therapy.

CHAPTER 2: MATERIALS AND METHODS

Cell Culture

Tumor Cells: Raji (American Type Culture Collection (ATCC), Manassas, VA) and NALM-6 (courtesy of David M. Nanus, Weill Cornell Medical College, New York, NY) tumor cell lines were maintained in RPMI 1640 (The Core Media Preparation Facility, Memorial Sloan Kettering Cancer Center (MSKCC), New York, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), nonessential amino acids, HEPES buffer (Gibco, Invitrogen, Waltham, MA), sodium pyruvate (Gibco), β-mercaptoethanol (Gibco), L-glutamine (2 mmol/L; Gibco), penicillin (100 U/mL; Gibco), and streptomycin (100 µg/mL; Gibco).

Artificial Antigen Presenting Cells (AAPC): NIH-3T3 mouse fibroblast AAPCs – 3T3($PSMA^+/CD80^+$) (Gong et al., 1999), 3T3($CD19^+/CD80^+$) (Brentjens et al., 2003), and 3T3($MUC\text{-}16^{ecto+}/CD80^+$) (Koneru et al., 2015) – have been previously published. The AAPCs were maintained in Dulbecco's Modified Eagle Medium (DMEM; The Core Media Preparation Facility, MSKCC, New York, NY) supplemented with 10% heat-inactivated donor calf serum (Thermo Fisher Scientific, Waltham, MA), L-glutamine (2 mmol/L; Gibco, Invitrogen, Waltham, MA), penicillin (100 U/mL; Gibco), and streptomycin (100 µg/mL; Gibco).

Retroviral Producer Cell Lines: 293 Gibbon ape leukemia virusaLV-9 19-28z, 19-28z/mIL-12, 19-28z/hIL-12, Pz1, Pz1/mIL-12, 4H11-28z, 4H11-28z/mIL-12, and 4H-1128z/hIL-12 were maintained in DMEM supplemented with 10% heat-inactivated

(FBS; Atlanta Biologicals, Norcross, GA), L-glutamine (2 mmol/L; Gibco, Invitrogen, Waltham, MA), penicillin (100 U/mL; Gibco), and streptomycin (100 µg/mL; Gibco).

T Cells: Human T cells were cultured in human T cell media – RPMI 1640 supplemented with 10% heat-inactivated FBS (The Core Media Preparation Facility, MSKCC), recombinant human IL-2 (100 IU/mL; *Proleukin*, Novartis, Basel, Switzerland), L-glutamine (2 mmol/L; Gibco), penicillin (100 U/mL; Gibco), and streptomycin (100 µg/mL; Gibco).

Generation of Retroviral Constructs

Generation of 19-28z (CD19-specific) (Brentjens et al., 2007), Pz1 (PSMA-specific) (Gong et al., 1999), and 4H11-28z (MUC16-specific) (Chekmasova et al., 2010) scFv-CD28-CD3ζ chain fusion proteins have been previously described. For CD19-specific CAR, we cloned the heavy (V_H)- and light (V_L)-chain variable regions from hybridoma cell line SJ25C1–derived cDNA by PCR and fused these coding regions with a $(Gly_3Ser)_4$-coding DNA fragment. The human CD8 leader peptide was ligated to the 5' end of the resulting scFv. The hinge and transmembrane coding regions of the human CD28 gene and the cytoplasmic domains of the human CD28 and CD3 ζ-chain were ligated to the 3' end of the scFv.

The 19-28z, Pz1, and 4H11-28z retroviral constructs were further modified to include a gene encoding the murine flexi-IL-12 (courtesy of Alan Houghton and Jedd Wolchok (Ferrone et al., 2006)) or human flexi-IL-12 (Wagner et al., 2004) with a serine-glycine repeat linker between the p35 and p40 domains with a CD8 leader peptide distal to the CAR gene and preceded by an internal ribosome entry site (IRES). The

resulting fusion genes were cloned into the modified moloney murine retroviral vector SFG (Rivière et al., 1995). Please refer to Figure 4.1 for a schematic diagram of 19-28z and 19-28z/mIL-12 constructs with the corresponding components of the CAR depicted in transduced CAR T cells.

293 H29 Transfection/293 GaLV-9 Transduction

VSV-G pseudotyped retroviral supernatants derived from transduced gpg29 fibroblasts were used to construct stable 293 GaLV-9 envelope-pseudotyped retroviral producing cell lines as described previously using polybrene (Gong et al., 1999). 293 H29 packaging cell line was plated at 30-50% confluence and $CaPO_4$ transfected with DNA encoding CAR construct using the *ProFection Mammalian Transfection System* (Promega, Madison, WI). The supernatant from the transiently transfected 293 H29 cell lines was collected every 24 hours for three days. Each day the viral supernatant along with polybrene (800 µg/mL; AmericanBio, Inc., Natick, MA) was added to a 30-50% confluent 293 GaLV-9 retroviral producing cell line for stable transduction. The transduced 293 GaLV-9 retroviral producing cell line was assessed for CAR expression by flow cytometry following staining with CAR-specific monoclonal goat anti-mouse phycoerythrin (PE)-conjugated antibody (Invitrogen, Waltham, MA) for CD19-targeted CAR, AlexaFluor647-conjugated Armenian hamster antibody that specifically binds to the 4H11-28z CAR (Memorial Sloan-Kettering Cancer Center Monoclonal Antibody Facility), and anti-PSMA fluorescein isothiocyanate (FITC)-conjugated antibody (Abcam, Cambridge, United Kingdom).

T Cell Isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors under Institutional Review Board approved protocol 95-091 using *BD Vacutainer CPT tubes* (Becton Dickinson, East Rutherford, NJ) or from a leukopak (New York Blood Center, New York, NY) and fractionated using density gradient centrifugation with *Accu-Prep™ Lymphocytes* (Axis-Shield, Dundee, Scotland). For isolation using *BD Vacutainer CPT Tubes* (Becton Dickinson), blood collected in CPT tubes was centrifuged at 1700 RCF (relative centrifugal force) for 20 min with no brake. The buffy coat was collected and washed twice with phosphate-buffered saline (PBS) at 1300 rpm centrifugation. The isolated PBMC was resuspended in human T cell media at 3 x 10^6 cells/mL concentration. For isolation using leukopak and *Accu-Prep™ Lymphocytes* (Axis-Shield), 35 mL of blood was transferred to 50 mL tubes and each tube was underlaid with 10 mL of lymphoprep. The tubes were centrifuged at 800 RCF for 20 min with no brake. The buffy coat was collected, washed as described above, and the isolated PBMC was resuspended in human T cell media at 3 x 10^6 cells/mL concentration.

$CD4^+CD25^{hi}Foxp3^+$ nTreg Isolation and Expansion

$CD4^+CD25^{hi}Foxp3^+$ natural Tregs (nTregs) were isolated from PBMCs using *Human $CD4^+CD25^{HIGH}$ T Cell Isolation Kit* (Stemcell Technologies, Vancouver, Canada) according to manufacturers' instructions. Using antibodies bound to magnetic beads, PBMCs were negatively selected with monoclonal antibodies targeting non-CD4 T cell antigens (CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCRγ/δ,

CD66b and glycophorin A), and then positively selected with CD25 antibody. Purity of nTregs was assessed with flow cytometry following staining with anti-human CD4, CD25, and FOXP3 antibodies.

The isolated nTregs were stimulated with *Dynabeads® Human T-Activator CD3/CD28 for T cell Expansion and Activation* (Gibco) at 1:1 bead:cell ratio and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, IL-2 (500 IU/mL every two to three days; *Proleukin*, Novartis), and rapamycin (100 ng/mL; Sigma Aldrich, St. Louis, MO) (Lee et al., 2011). Expanded nTregs were reassessed for FOXP3 expression after three weeks of expansion by flow cytometry following staining with anti-human CD4, CD25, and FOXP3 antibodies.

Retroviral Gene Transfer of T Cells and nTregs

T Cells: Gene transfer of retroviral vector into effector T cells has been previously described (Brentjens et al., 2007; Lee et al., 2009)**.** T cells were activated with phytohemagglutinin (2 µg/ml; Sigma Aldrich) and IL-2 (100 IU/ml; *Proleukin*, Novartis) for 48 hours and transduced in 6-well non–tissue culture plates (Becton Dickinson) coated with 15 µg/ml retronectin (Takara Biomedicals, Otsu, Japan). For three consecutive days, fresh retroviral producer supernatant was added to T cells daily followed by centrifugation at 3,200 rpm for one hour at 30°C. The transduction efficiency of CAR T cell was assessed by flow cytometry following staining with CAR-specific monoclonal goat anti-mouse PE-conjugated antibody (Invitrogen, Waltham, MA). For *in vivo* tumor models, CAR T cells were expanded by re-stimulating with 3T3($CD19^+/CD80^+$), 3T3($PSMA^+/CD80^+$), or 3T3($MUC\text{-}16^{ecto+}/CD80^+$) AAPC to

generate sufficient quantity of tumor-specific T cells (Kim et al., 2004; Latouche and Sadelain, 2000)**.**

nTregs: Isolated nTregs were activated with *Dynabeads® Human T-Activator CD3/CD28 for T cell Expansion and Activation* (Gibco, Invitrogen) at 1:1 bead:cell ratio and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, IL-2 (500 IU; *Proleukin*) and rapamycin (100 ng/mL) for 48 hours, (Battaglia et al., 2005; Strauss et al., 2007) and transduced using the same protocol of T cell transduction. The transduction efficiency in nTregs was assessed by flow cytometry following staining with CAR-specific monoclonal goat anti-mouse PE-conjugated antibody (Invitrogen).

T Cell Proliferation

Carboxyfluorescein succinimidyl ester (CFSE) Proliferation Assay: 1 x 10^6 CAR T cells were labeled with 5 µmol/L CFSE (Invitrogen) and stimulated with anti-CD3/CD28 beads (Invitrogen) at 1:3 bead:cell ratio in the presence or absence of recombinant murine IL-12 (mIL-12; 10 ng/mL) for 72 hours in 24-well tissue culture-coated plates (Becton Dickinson, East Rutherford, NJ) in the absence of exogenous IL-2 followed by flow cytometry analysis. Percentage of T cell proliferation was assessed by gating on the population with diluted CFSE expression compared to unstimulated T cells.

For nTreg suppression experiments, 5 x 10^5 CFSE-labeled CAR T cells were co-cultured with titrated numbers of purified autologous $CAR^+CD4^+CD25^+Foxp3^+$ natural Tregs (nTregs) (Oberg et al., 2006)**.** T cell co-cultures were stimulated with anti-

CD3/CD28 beads (Invitrogen) at 1:3 bead:cell ratio and proliferation was assessed by flow cytometry at 72 hours.

CD4 and CD8 Proliferation: CD4 and CD8 T cells were isolated from T cells previously transduced with CAR using *Dynabeads Untouched Human CD4 T cells* and *Dynabeads Untouched Human CD8 T cells* (Invitrogen) magnetic bead isolation kits, respectively. For CD4 T cell isolation, T cells were negatively selected with antibodies targeting CD8, CD14, CD16, CD19, CD36, CD56, CDw123, and glycophorin A. For CD8 T cell isolation, T cells were negatively selected with antibodies targeting CD4, CD14, CD16, CD19, CD36, CD56, CDw123, and glycophorin A. 1 x 10^6 CD4 T and CD8 T cells were stimulated with anti-CD3/CD28 beads (Gibco) at 1:3 bead:cell ratio. Exogenous mIL-12 (10 ng/mL; R&D System, Inc., Minneapolis, MN) was added to the media daily for the duration of the experiment. Total cell number of viable cells was enumerated by GuavaViaCount (Millipore, Billerica, MA) on days 0, 3, 7, 10, 14, and 21 after stimulation. Fold expansion of cells were calculated by dividing the cell counts at the specified time points by the initial number of viable cells.

NALM-6 and Raji Tumor-Stimulated Proliferation: 1 x 10^6 CAR^+ T cells were labeled with *CellVue Claret Far Red Fluorescent dye* (Sigma Aldrich) and co-cultured with 1 x 10^6 NALM-6 or Raji tumor cells in 12-well tissue culture-coated plates (Becton Dickinson). Total cell counts of viable cells were enumerated by GuavaViaCount (Millipore) and flow cytometry analysis of CD3, CD19, and CAR expression was assessed on days 0, 3, 7, 10, 14, and 21 after co-culture. Total CAR^+ T cell counts were calculated at the specified time points and the relative fold expansion

was calculated by dividing the cell counts at the specified time points by the initial number of CAR^+ T cells.

Proliferation with Repeated Antigen Exposure: 9 x 10^6 CAR^+ T cells were cultured in three wells of 70% confluent 3T3($CD19^+$/$CD80^+$) AAPCs in 6-well tissue culture-coated plates. Every seven days, CAR expression was assessed by flow cytometry and total cell counts were enumerated by using a hemocytometer and nonviable cells were excluded by trypan blue. 9 x 10^6 CAR^+ T cells recovered from the previous week were re-stimulated with 3T3($CD19^+$/$CD80^+$) AAPCs. Total CAR^+ T cell counts were calculated at the specified time points and the relative fold expansion was calculated by dividing the cell counts at the specified time points by the initial number of CAR^+ T cells.

T Cell Cytotoxicity Assays

Chromium Release Assay: Cytolytic capacity of CAR T cells was assessed using a standard chromium release assay as described previously (Gong et al., 1999)**.** Transduced CAR T cells were incubated for 4 hours at different effector to target (E:T) ratios with ^{51}Cr-labeled target cells at 37°C. The amount of ^{51}Cr in the supernatant was determined with a Top Count automated gamma counter (Perkin Elmer, Waltham, MA). Percentage of specific ^{51}Cr release was calculated by (^{51}Cr release – spontaneous release)/(maximum ^{51}Cr release – spontaneous release) x 100.

Cytotoxic Activity at 72 hours: 1 x 10^6 CAR^+ T cells were labeled with *CellVue Claret Far Red Fluorescent dye* (Sigma Aldrich) and co-cultured with 1 x 10^6 NALM-6 tumor cells in 12-well tissue culture-coated plates. Total cell count was assessed using

a hemocytometer with trypan blue exclusion of nonviable cells and CD19 and CD3 expression were assessed by flow cytometry at 72 hours after co-culture. Total number of NALM-6 tumor cells is calculated based on the percentage of $CD19^+$ cells and absolute cell counts.

nTreg Suppression Assays: To assess nTreg-mediated inhibition of CAR T cell cytotoxicity, 1 x 10^6 CAR T cells were co-cultured with 1 x 10^6 nTregs in RPMI media for 24 hours in 12-well tissue culture treated plates. 1 x 10^6 Raji tumor cells were subsequently added to the culture. Tumor lysis was subsequently assessed by flow cytometry to detect residual $CD19^+$ tumor cells.

Cytokine Detection Assay

Cytokine levels in tissue culture supernatant were quantified using multiplex Human Cytokine Detection System (Millipore) in conjunction with the Luminex IS 100 system and IS 2.2 software (Luminex Corporation, Austin, TX) according to the manufacturer's instructions.

Mouse Tumor Models

NALM-6 B-ALL tumor model: 8- to 12-week-old Fox Chase SCID-Beige (CB17.Cg-Prkdc^{scid}Lyst^{bg-J}/Crl) mice (Charles Rivers, Wilmington, MA) were injected with 1 x 10^6 NALM-6 tumor cells intravenously (i.v.) followed by a single injection (i.v.) of 1 x 10^7 CAR T cells on day 6. Mice were monitored clinically for disease progression and were euthanized when disease was evident as characterized by ruffled fur, weight loss, and/or hind limb paralysis. In a separate study to assess CAR T cell

survival, mice were injected (i.v.) with 1 x 10^6 NALM-6 tumor cells followed by a single injection (i.v.) of 1 x 10^7 CAR T cells on day 14. Retro-orbital blood samples were then collected 7 days and 14 days following CAR T cell infusion to assess persistence of CAR T by flow cytometry analysis of CD3 and CAR expression.

nTreg suppression tumor model: 5 x 10^5 Raji tumor cells were infused (i.v.) on day 1, 1 x 10^7 CAR^+ nTregs were infused (i.v.) on day 5, and 1 x 10^7 CAR T cells were infused (i.v.) on day 6 (Lee et al., 2011)**.** All *in vivo* studies were performed in accordance with the Institutional Animal Care and Use Committee at Memorial Sloan-Kettering Cancer Center (approved protocol #00-05-065).

Bioluminescent Imaging of NALM-6 Tumors *in vivo*: Bioluminescence imaging was done using Xenogen IVIS Imaging System (Perkin Elmer) with Living Image 4.4 software (Perkin Elmer) for acquisition of imaging data sets. Mice were injected intraperitoneally (i.p.) with 150 mg/kg D-luciferin (Perkin Elmer) resuspended in 200 µL PBS. Five minutes later, mice were anesthetized with 2% isoflurane and imaged. Image acquisition of ventral surface was performed with a 25 cm field of view at medium binning level for 30 s exposure time or 1 s exposure time when the bioluminescent intensity reached saturation.

Flow Cytometry

Flow cytometry was performed using BD LSRII (Becton Dickinson) or Gallios Flow Cytometer (Beckman Coulter, Brea, CA) and analyzed with Flowjo10 software. Raji and NALM-6 tumor cells were stained with anti-human CD19 antibody (Invitrogen). For phenotypic characterization, human T cells were stained with anti-

human CD8 (Invitrogen), CD4, CD45RA, CCR7, and CD62L (eBioscience, Santa Clara, CA) antibodies. For granzyme B and perforin analyses, T cells were permeabilized with BD Cytofix/Cytoperm Kit and stained with anti-human granzyme B and perforin antibodies (eBioscience). Analysis of T cell exhaustion was assessed by staining CAR T cells with anti-human PD-1 and TIM-3 antibodies (eBioscience). Analysis of nTregs was assessed by staining with anti-human CD4, CD25, CD127, and FOXP3 antibodies (eBioscience). CAR expression was assessed using CAR-specific monoclonal goat anti-mouse phycoerythrin (PE)-conjugated antibody (Invitrogen) or PE-conjugated CAR-specific 19E3 antibody (MSKCC monoclonal antibody core facility). All cells were stained with Fixable Viability Dye eFluor®450 (eBioscience).

Statistical Analysis

All analyses were calculated using GraphPad Prism 6 software. Log rank Mantel-Cox test was used to analyze survival studies, and Mann-Whitney test was used for other analyses.

CHAPTER 3: EFFECT OF EXOGENOUS IL-12 ON CAR T CELLS

Exogenous IL-12 enhances 19-28z CAR T cell proliferation

To evaluate the therapeutic benefit of IL-12 in the context of CD19-specific CAR T cells, we retrovirally transduced human peripheral blood mononuclear cells (PBMCs) with 19-28z CAR and cultured the cells in the presence or absence of exogenous recombinant murine IL-12 (mIL-12). Due to the cross-reactivity of mIL-12 with human IL-12 receptor, we reasoned that mIL-12 would be appropriate for studying human T cells both *in vitro* and in xenotransplant mouse models (Schoenhaut et al., 1992; Trinchieri, 1994). We stimulated 19-28z CAR T cells with anti-CD3/CD28 beads for 72 hours in the presence or absence of mIL-12 and monitored proliferation of the CAR T cells. Utilizing a CFSE proliferation assay, we observed a statistically significant increase in proliferation in 19-28z CAR T cells treated with mIL-12 as compared to the absence of mIL-12 stimulation ($p<0.05$, $n=7$; Figure 3.1A). We further examined the effect of mIL-12 on the expansion of the CD4 and CD8 subsets of 19-28z CAR T cells over a three week period. We separated 19-28z CAR T cells into CD4 and CD8 populations and stimulated the cells with anti-CD3/CD28 beads in the presence or absence of exogenous mIL-12. In both the CD4 and CD8 populations, the 19-28z CAR T cells that were treated with mIL-12 exhibited significantly increased expansion as compared to 19-28z CAR T cells cultured without mIL-12 ($p<0.01$, $n=3$; Figure 3.1B).

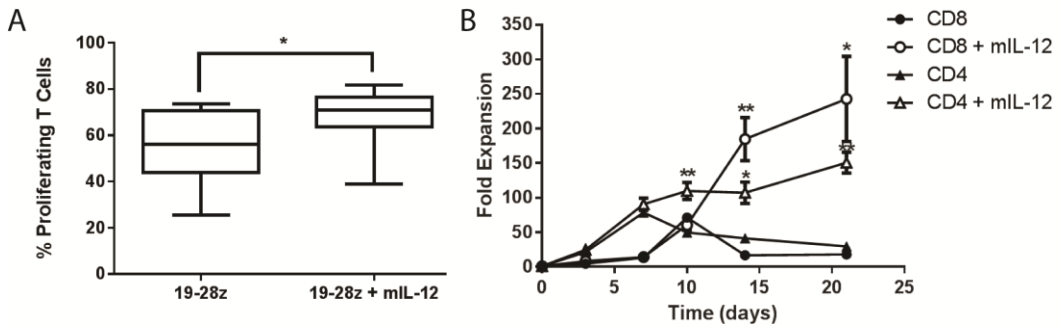

Figure 3.1 Exogenous mIL-12 enhances 19-28z CAR T cell proliferation
(A) CFSE proliferation assay showing percent proliferating CAR T cells after three days of anti-CD3/CD28 bead stimulation in the presence or absence of exogenous mIL-12. *p<0.05, n=7. (B) Expansion of 19-28z CD8 (circle) and CD4 (triangle) CAR T cells after anti-CD3/CD28 bead stimulation in the presence (unshaded) or absence (shaded) of exogenous mIL-12. Fold expansion of CAR T cells determined by enumeration with GuavaViaCount of viable cells and flow cytometry analysis of CAR expression. For comparison between mIL-12 and non-IL-12 cultured groups, *p<0.05, **p<0.01, n=3.

Exogenous IL-12 enhances cytotoxic activity of 19-28z CAR T cells

We next studied the effect of IL-12 on the cytotoxic potential of 19-28z CAR T cells. We first examined the expression of two molecules critical for the cytotoxic activity of cytotoxic T lymphocytes (CTLs) – granzyme B (GzmB) and perforin (Pfn). Perforin secreted by CTLs binds to the plasma membrane of target cell and oligomerizes in a $Ca2^{+}$-dependent manner to form large transmembrane pores that enable diffusion of granzymes into the target cell cytosol (Voskoboinik et al., 2015). Granzymes in turn induce apoptosis in the target cell by directly activating the pro-apoptotic factor Bid or indirectly through caspase 8 activation (Barry et al., 2000). Granzyme B is the most dominant pro-apoptotic granzyme which can induce apoptosis rapidly even in the

context of low perforin levels (Voskoboinik et al., 2015). We observed that a higher percentage of 19-28z CAR T cells express granzyme B ($p<0.05$, n=4) and perforin ($p<0.05$, n=3) in the presence of exogenous mIL-12 as compared to 19-28z CAR T cells cultured in the absence of exogenous mIL-12 (Figure 3.2).

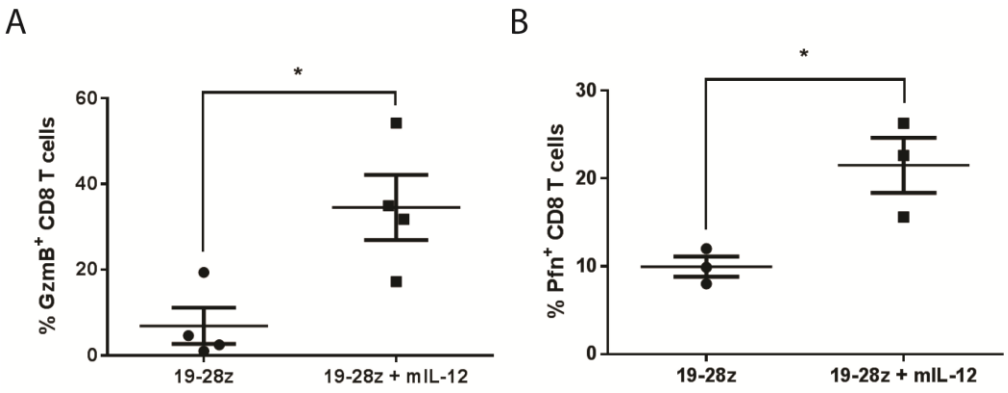

Figure 3.2 Exogenous mIL-12 enhances granzyme B and perforin expression in 19-28z CAR T cells
19-28z CAR T cells were cultured in the presence or absence of exogenous mIL-12 for seven days and assessed for the expression of (A) granzyme B (GzmB) and (B) perforin (Pfn) by flow cytometry. Data showing percentage of 19-28z CAR T cells expressing GzmB and Pfn among the CD8 population. *$p<0.05$, n=4 (A), n=3 (B).

Exogenous IL-12 induces a distinct pro-inflammatory Th1/Tc1-like phenotype in 19-28z CAR T cells

To determine the effect of mIL-12 on 19-28z CAR T cell function, we compared the cytokine secretion levels of 19-28z CAR T cells cultured in the presence and absence of exogenous mIL-12 after 48 hours of anti-CD3/CD28 bead stimulation. The mIL-12-cultured 19-28z CAR T cells were characterized by a predominantly pro-inflammatory Th1/Tc1-type immune response. In both the CD4 and CD8 populations, a significantly

enhanced production of the Th1 cytokine IFN-γ (CD8: $p=0.0149$, $n=3$; CD4: $p<0.0001$) was secreted from 19-28z CAR T cells cultured in the presence of exogenous mIL-12 as compared to 19-28z CAR T cells cultured in the absence exogenous mIL-12 (Figure 3.3). At the same time, the production of the Th2 cytokines IL-5 (CD8: $p=0.0001$, $n=3$; CD4: $p=0.0002$, $n=3$) and IL-13 (CD8: $p=0.0002$, $n=3$; CD4: $p<0.0001$, $n=3$) was reduced in the mIL-12-cultured 19-28z CAR T cells as compared to 19-28z CAR T cells cultured in the absence of mIL-12 (Figure 3.3). Among the CD4 population, the Th17 cytokine IL-17A was also significantly reduced in the mIL-12-cultured 19-28z CAR T cells as compared to the 19-28z CAR T cells cultured in the absence of mIL-12 ($p<0.0001$, $n=3$; Figure 3.3).

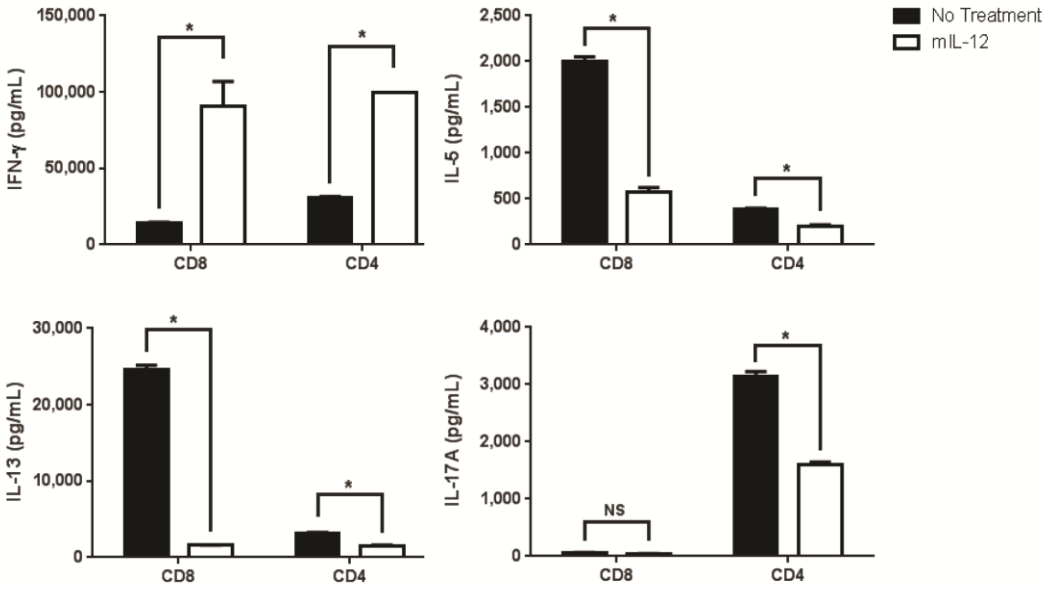

Figure 3.3 Exogenous IL-12 induces 19-28z CAR T cells towards a distinct pro-inflammatory Th1/Tc1-like phenotype

19-28z CAR T cells were stimulated with anti-CD3/CD28 beads in the presence or absence of exogenous mIL-12 for 48 hours and the supernatant was assessed by luminex. Secretion of IFN-γ, IL-5, IL-13, and IL-17A in CAR T cells after stimulation. *$p<0.05$, n=3; NS, p=not significant.

CHAPTER 4: IL-12-SECRETING CAR T CELLS

Generation of IL-12-secreting CAR T cells

Having established the beneficial effect of exogenous mIL-12 on CAR T cell proliferation, cytotoxic potential, and cytokine production, we reasoned that CAR T cells further modified to constitutively express mIL-12 may have similar increased proliferation and enhanced *in vitro* and *in vivo* anti-tumor function. Furthermore, generating IL-12-secreting CAR T cells allows targeted delivery of IL-12 into the tumor microenvironment, reducing the cytotoxic effect associated with systemic recombinant IL-12 administration. We inserted an IRES element followed by a fusion gene encoding the p35 and p40 subunits of murine IL-12 (flexi-IL-12) (Ferrone et al., 2006) into the 19-28z retroviral CAR construct (Figure 4.1). Similarly, we generated two other IL-12-secreting control CAR constructs by inserting mIL-12 in the constructs encoding the prostate-specific membrane antigen (PSMA)-specific CAR (Pz1) and the ovarian cancer antigen $MUC16^{ecto}$ (4H11-28z) to generate Pz1/mIL-12 and 4H11-28z/mIL-12 CAR constructs, respectively.

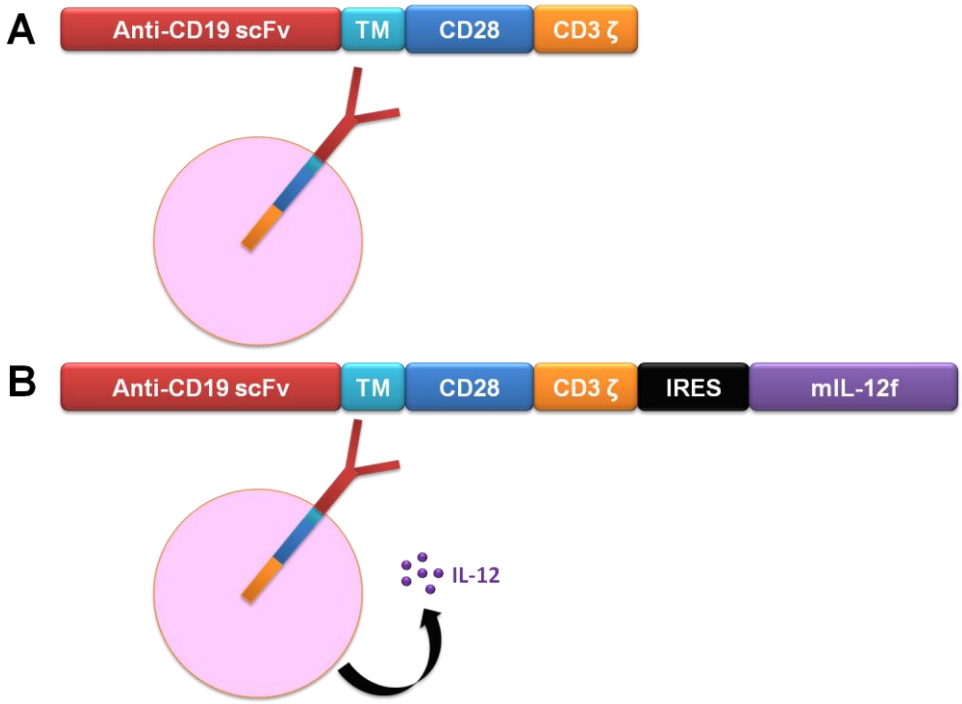

Figure 4.1 Chimeric antigen receptor retroviral construct
Schematic diagram of (A) 19-28z and (B) 19-28z/mIL-12 retroviral constructs. *scFv, single chain variable fragment; TM, transmembrane domain; IRES, internal ribosome entry site; mIL-12f, murine flexi-IL-12.*

19-28z/mIL-12 CAR T cells secrete functional murine IL-12

The 19-28z/mIL-12 retroviral CAR construct was transduced into a stable 293-GaLV9 retroviral producer cell line. The 19-28z/mIL-12 retroviral producer cell line expressed the CAR (Figure 4.2A) and secreted mIL-12 after 24 hours of culture (Figure 4.2B). To confirm that the mIL-12 secreted by the 19-28z/mIL-12 producer cell line is biologically active, we cultured PBMCs with the supernatant from the 19-28z or 19-28z/mIL-12 producer cell lines for 24 hours and assessed the level of IFN-γ secretion as it has been established that mIL-12 induces IFN-γ secretion (Chan et al., 1991).

Compared to cells stimulated with 19-28z producer supernatant, PBMCs cultured with 19-28z/mIL-12 producer supernatant secreted a significantly greater amount of IFN-γ ($p<0.05$, n=4) (Figure 4.2C).

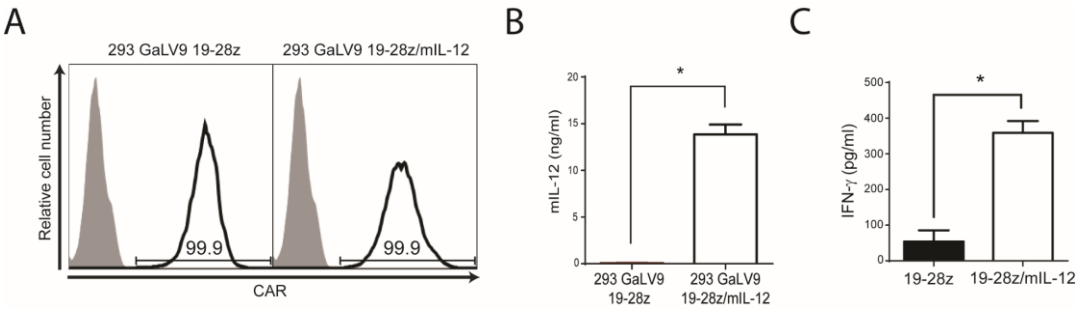

Figure 4.2 GaLV-9 19-28z/mIL-12 retroviral producer cell line validation
(A) CAR expression in GaLV-9 19-28z and 19-28z/mIL-12 viral producer cell lines as assessed by flow cytometry following staining with monoclonal antibody that specifically binds the CAR-specific monoclonal goat anti-mouse PE-conjugated antibody. (B) mIL-12 expression in GaLV-9 CAR viral producer cell line was assessed by luminex after 24 hours of cell culture (*$p<0.05$, n=4). (C) GaLV-9 19-28z/mIL-12 viral producer cell line secreted functional mIL-12. PBMCs were stimulated with supernatant from GaLV-9 19-28z or 19-28z/mIL-12 viral producer cell line for 24 hours, and the supernatant from the PBMC cultures were analyzed for secretion of IFN-γ by luminex (*$p<0.05$, n=4).

19-28z/mIL-12 CAR T cells are characterized by a unique pro-inflammatory Th1/Tc1-like phenotype

We next transduced human T cells to express both the 19-28z CAR and mIL-12. Comparable levels of retroviral gene transfer was observed following transduction with either 19-28z or 19-28z/mIL-12 vector (Figure 4.3A). As expected, only T cell modified with the 19-28z/mIL-12 vector secreted mIL-12 (Figure 4.3B).

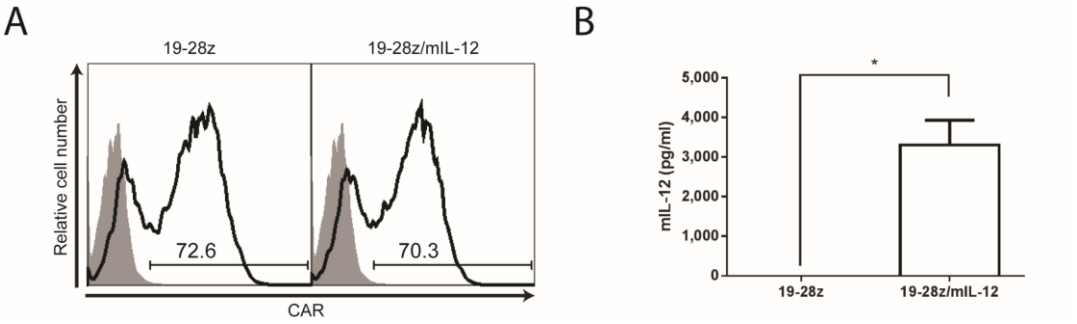

Figure 4.3 19-28z/mIL-12 CAR T cells secrete mIL-12
(A) Comparable 19-28z and 19-28z/mIL-12 CAR expression in T cells after gene transfer as assessed by flow cytometry. (B) High levels of mIL-12 detected in supernatant from 19-28z/mIL-12 CAR T cells but absent in 19-28z CAR T cells at 24 hours after anti-CD3/CD28 bead stimulation as determined by luminex (*$p<0.01$, n=5).

To determine the type of immune response 19-28z/mIL-12 CAR T cells produced in the context of tumor cells, we compared the cytokine levels secreted from 19-28z and 19-28/mIL-12 CAR T cells after 24 hours of culture with NALM-6 or Raji tumors. In the context of NALM-6, we demonstrated that 19-28z/mIL-12 CAR T cells display a Th1/Tc1-like phenotype as characterized by elevated levels of IFN-γ ($p<0.05$, n=4) and lower levels of IL-5 ($p<0.01$, n=5), IL-13 ($p<0.01$, n=5), and IL-17A ($p<0.05$, n=4) compared to 1928z CAR T cells (Figure 4.4A). Stimulation of 19-28z or 19-28z/mIL-12 CAR T cells with Raji tumors resulted in similar levels of cytokine secretion (Figure 4.4B).

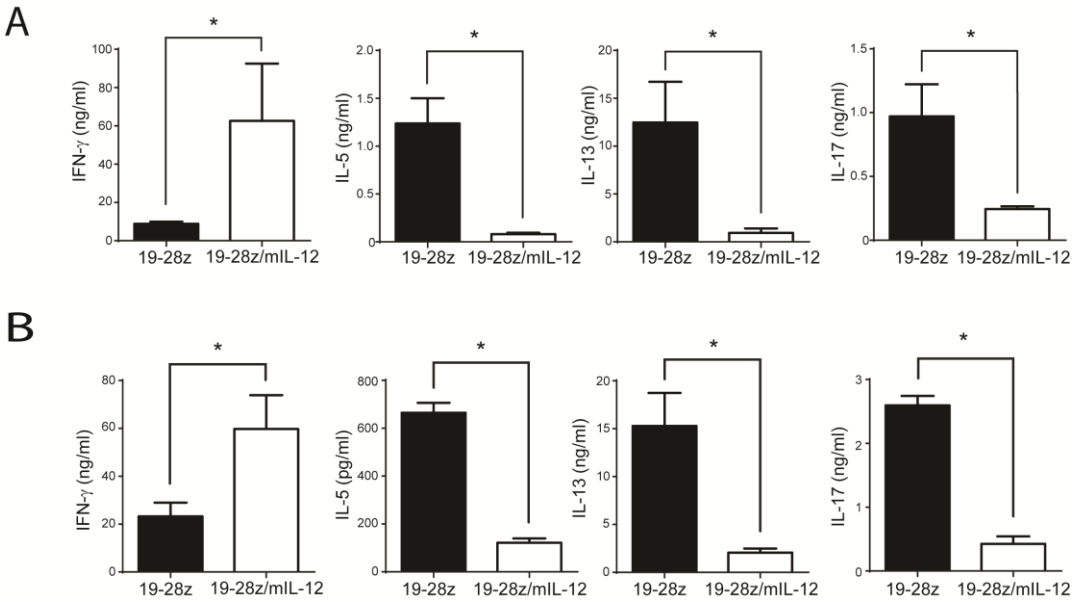

Figure 4.4 19-28z/mIL-12 CAR T cells have a Th1/Tc1-like phenotype
Secretion of IFN-γ, IL-5, IL-13, and IL-17 in CAR T cells after CAR T cells were stimulated with (A) NALM-6 or (B) Raji tumor cells at 1:1 ratio for 24 hours as assessed by luminex. *$p<0.05$, n=5.

19-28z/mIL-12 CAR T cells have enhanced proliferation compared to 19-28z CAR T cells and display a T_{CM}-like phenotype

We next assessed the proliferative capacity of CAR T cells by re-stimulating once with NALM-6 tumor cells and quantifying the number of viable CAR T cells over a three week period. 19-28z/mIL-12 CAR T cells have an increased expansion compared to 19-28z CAR T cells (Figure 4.5A, left panel). This increased expansion can be attributed to increased proliferation that can be observed as early as ten days after stimulation ($p=0.05$, n=3) (Figure 4.5A, right panel). Stimulation of CAR T cells with Raji tumor cells yielded similar enhanced proliferation in the 19-28z/mIL-12 CAR T cells as compared to 19-28z CAR T cells (Figure 4.5B). Furthermore, we observed that

at ten days following gene transfer, a significantly higher percentage of 19-28z/mIL-12 CAR T cells are characterized by a $CCR7^{+}CD62L^{hi}$ central memory (T_{CM})-like phenotype ($p<0.01$, n=7) as compared to the 19-28z CAR T cell population (Figure 4.6).

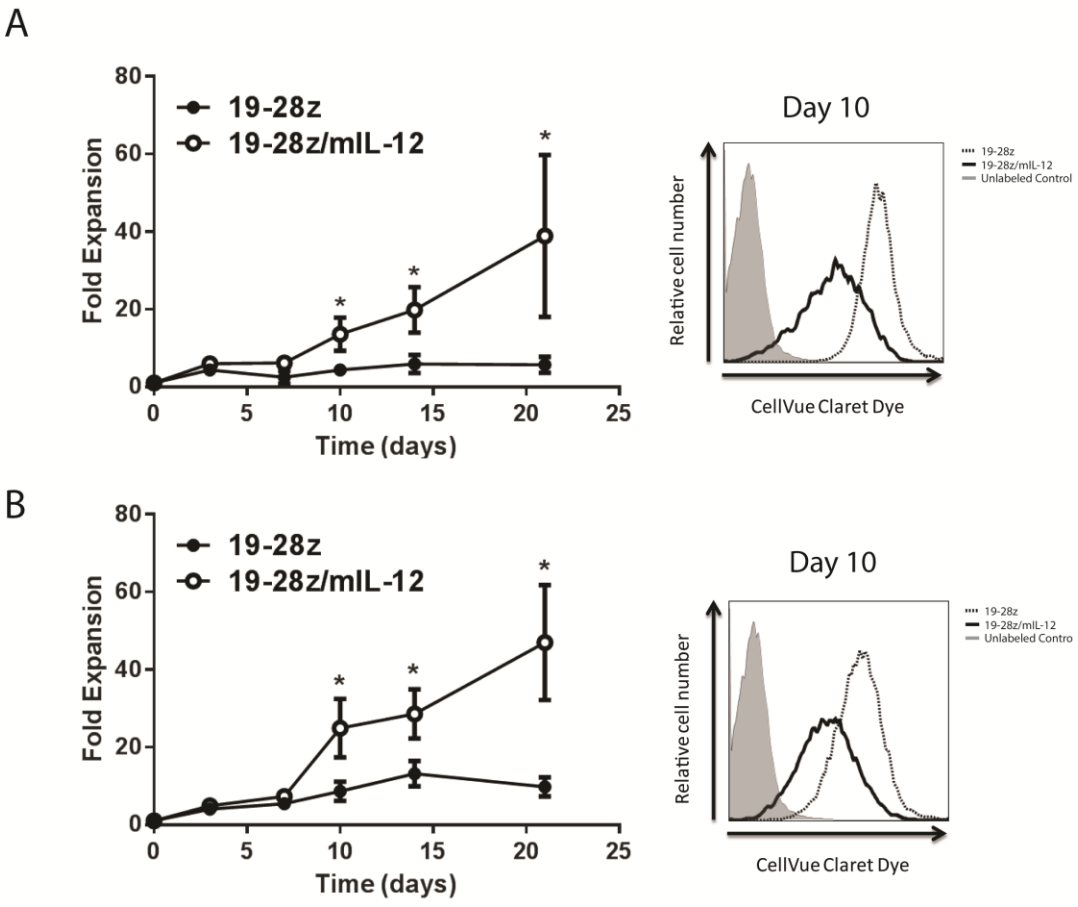

Figure 4.5 19-28z/mIL-12 CAR T cells have enhanced proliferation as compared to 19-28z CAR T cells
19-28z/mIL-12 CAR T cells have increased proliferation compared to 19-28z CAR T cells in the context of (A) NALM-6 and (B) Raji tumors. CAR T cells were labeled with CellVue Claret Far Red Fluorescent dye and co-cultured with Raji cells at a ratio of 1:1. Fold expansion (left panel) of CAR T cells determined by enumeration with GuavaViaCount of viable cells and flow cytometry analysis of CAR expression. Flow cytometry plot displays representative CellVue Claret Dye dilution on day 10 (right panels). For all time points, *p=0.05, n=3.

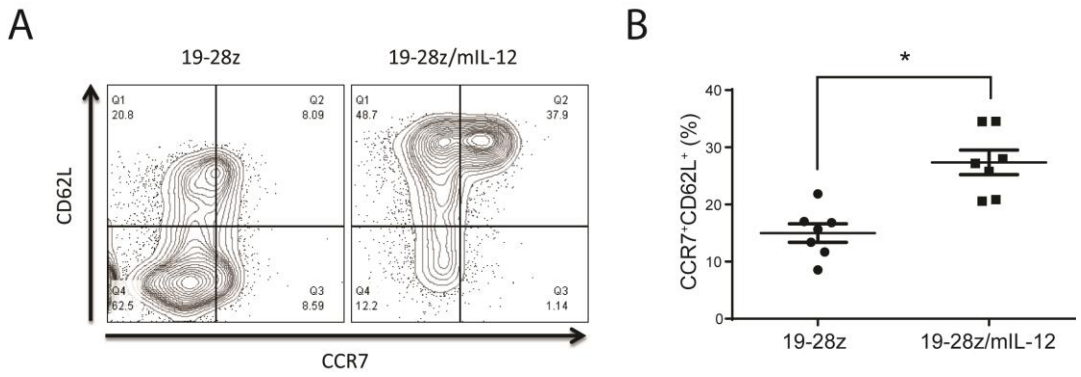

Figure 4.6 19-28z/mIL-12 CAR T cells express both CCR7 and CD62L
Higher percentage of $CCR7^{+}CD62L^{hi}$ T cells in 19-28z/mIL-12 CAR T cell population compared to 19-28z CAR T cell population observed at 10 days after initial gene transfer as assessed by flow cytometry. (A) Representative FACS plot of CCR7 and CD62L expression. (B) Percentage of $CCR7^{+}CD62L^{+}$ T cells. *$p<0.01$, n=7.

In vivo, CAR T cells may be prone to prolonged antigen exposure. To assess the long-term proliferative potential of 19-28z/mIL-12 CAR T cells under such conditions, we tracked the expansion of 19-28z and 19-28z/mIL-12 CAR T cells after repeated weekly stimulation with 3T3($CD19^{+}/CD80^{+}$) artificial antigen presenting cells (AAPCs). The 19-28z CAR T cells stopped expanding after the 3rd stimulation, while 19-28z/mIL-12 CAR T cells continued to expand after the 4th stimulation (Figure 4.7).

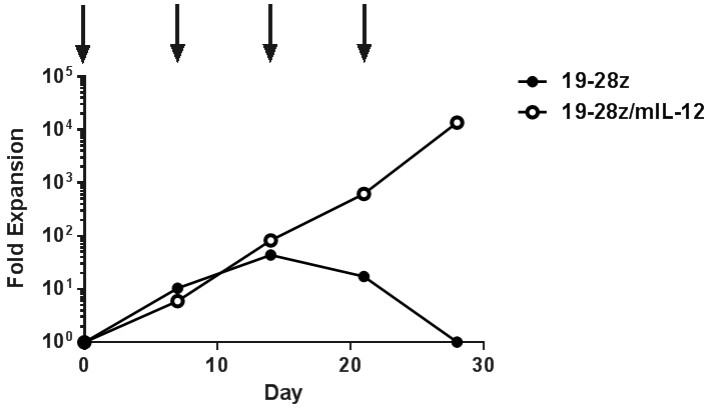

Figure 4.7 19-28z/mIL-12 CAR T cells retain proliferative capacity after repeated antigen exposure
$3x10^6$ 19-28z or 19-28z/mIL-12 CAR T cells were stimulated with 3T3($CD19^+/CD80^+$) AAPC weekly as indicated by black arrows. Fold expansion of CAR T cells determined by enumeration with GuavaViaCount of viable cells and flow cytometry analysis of CAR expression. Data represent two independent experiments.

IL-12 has been associated with the induction of exhaustion in T cells in the context of follicular B cell non-Hodgkin's lymphoma tumor (Yang et al., 2012). Exhaustive T cells are characterized by elevated PD-1 and TIM-3 expression (Crespo et al., 2013). To determine whether 19-28z/mIL-12 CAR T cells exhibits a more exhausted phenotype, we checked the expression of PD-1 and TIM-3 in CAR T cells that have been stimulated twice with 3T3($CD19^+/CD80^+$) AAPCs. However, we did not observe any significant difference in the percentage of $PD\text{-}1^+TIM\text{-}3^+$ cells between 19-28z and 19-28z/mIL-12 CAR T cell populations (Figure 4.8).

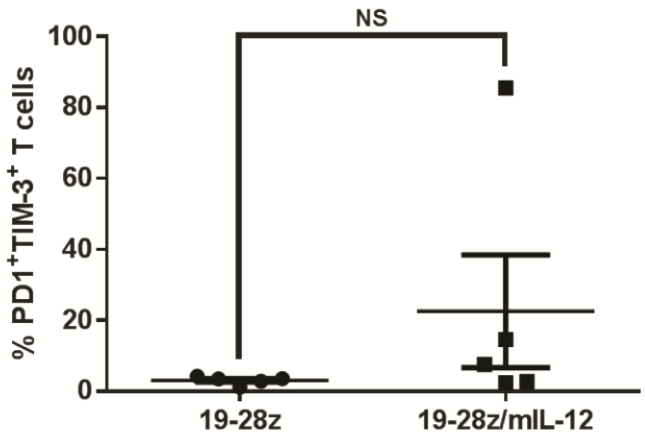

Figure 4.8 PD-1 and TIM-3 expression in CAR T cells
19-28z and 19-28z CAR T cells were stimulated twice with 3T3($CD19^+$/$CD80^+$) AAPC over two weeks. The percentage of $PD\text{-}1^+TIM\text{-}3^+$ cells was assessed by flow cytometry. NS, p=not significant, n=5.

19-28z/mIL-12 CAR T cells have enhanced cytotoxic activity compared to 19-28z CAR T cells

We next compared the expression of granzyme B and perforin in 19-28z and 19-28z/mIL-12 CAR T cells. Production of granzyme B ($p<0.0001$, n=5) and perforin ($p<0.0001$, n=3) was observed in a higher percentage of 19-28z/mIL-12 CAR T cells when compared to that of 19-28z CAR T cells indicating a potentially greater cytotoxic capacity (Figure 4.9A-B). As expected, 19-28z CAR T cells can effectively lyse NALM-6 tumors compared to control Pz1 CAR T cells (Figure 4.9C). We did not observe any advantage in the ability of 19-28z/mIL-12 CAR T cells to kill NALM-6 tumor cells in a 4 hour ^{51}Cr-release assay (Figure 4.9C). However, there was a significant decrease in overall NALM-6 tumor cell number ($p<0.01$, n=7) following 72 hour co-culture with

19-28z/mIL-12 CAR T cells at a 1:1 effector:target (E:T) ratio when compared to co-culture with 19-28z CAR T cells as assessed by flow cytometry (Figure 4.9D).

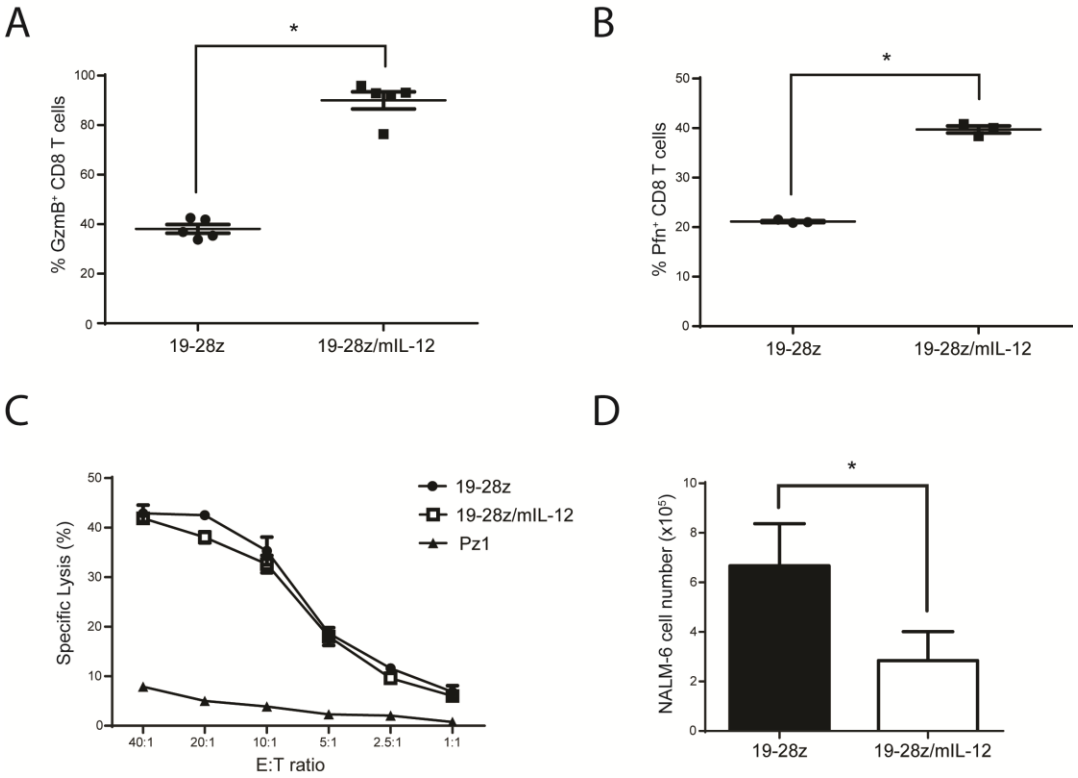

Figure 4.9 19-28z/mIL-12 CAR T cells have enhanced cytotoxic capacity compared to 19-28z CAR T cells

(A) 19-28z/mIL-12 CAR T cells express significantly more granzyme B compared to 19-28z CAR T cells as assessed by intracellular flow cytometry (*$p<0.0001$, n=5). (B) 19-28z/mIL-12 CAR T cells express significantly more perforin as compared to 19-28z CAR T cells as assessed by intracellular flow cytometry (*$p<0.0001$, n=3). (C) 19-28z and 19-28z/mIL-12 CAR T cells lyse NALM-6 tumor cells at similar levels in a four hour ^{51}Cr release assay. Data represent three independent experiments. (D) 19-28z/mIL-12 CAR T cells have enhanced cytotoxic activity as compared to 19-28z CAR T cells following 72 hours co-culture with NALM-6 tumor at 1:1 E:T ratio as assessed by enumeration with GuavaViaCount of viable cells and flow cytometry analysis of CD19 expression. (*$p<0.01$, n=7).

CHAPTER 5: EFFICACY OF IL-12-SECRETING CAR T CELLS *IN VIVO*

19-28z/mIL-12 CAR T cells eradicate systemic NALM-6 pre-B cell ALL tumors in SCID-Beige mice

We previously demonstrated that NALM-6 tumor bearing SCID beige mice have enhanced survival when treated with 19-28z CAR T cells as compared to treatment with first generation 19z1 CAR T cells. However, such therapeutic benefit accounted for only 44% of the treated mice and only after four weekly 19-28z CAR T cell infusions with the first infusion administered one day after tumor injection (Brentjens et al., 2007). Considering the effects of mIL-12 in enhancing T cell proliferation, cytotoxic activity, and promoting a T_{CM}-like phenotype in CAR T cells, we investigated the ability of 19-28z/mIL-12 CAR T cells to eradicate NALM-6 tumors in SCID/Beige mice. We infused 19-28z/mIL-12 or 19-28z CAR T cells seven days after tumor inoculation and monitored the mice for tumor progression. As controls, we also monitored the progression of mice infused with Pz1 or Pz1/mIL-12 CAR T cells targeting to the irrelevant PSMA antigen. All of the mice treated with control Pz1 CAR T cells died by day 29 (Figure 5.1). Furthermore, none of the Pz1/mIL-12 CAR T cell-treated mice survived beyond day 28, suggesting that mIL-12 secretion by non-tumor-targeting CAR T cells does not have any appreciable anti-tumor effect against NALM-6 tumors in this model. While 19-28z CAR T cell-treated mice exhibited delayed tumor progression compared to Pz1 ($p<0.0001$) and Pz1/mIL-12 ($p<0.0001$) control groups, all but one (6.67%) mouse eventually died of disease (Figure 5.1). Compared to the 19-28z CAR T cell-treated mice, the mice treated with 19-28z/mIL-12 CAR T cells had significantly

enhanced survival ($p<0.0001$) in which 93.3% of the mice survived and remained tumor-free on day 60.

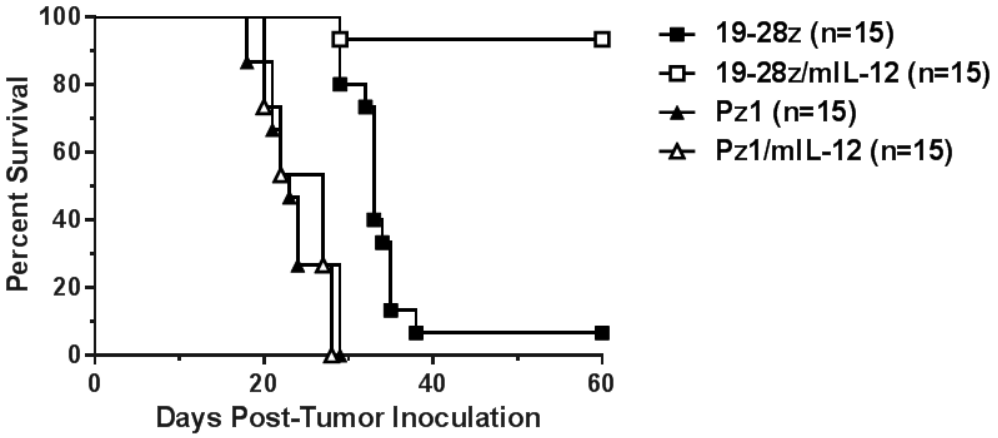

Figure 5.1 19-28z/mIL-12 CAR T cells eradicate systemic NALM-6 pre-B cell ALL tumors in SCID-Beige mice
19-28z/mIL-12 CAR T cell treatment enhances survival compared to 19-28z CAR T cell-treated mice. SCID-Beige mice were inoculated (i.v.) with $1x10^6$ NALM-6 tumor cells on day 0 and $1x10^7$ CAR T cells on day 6. Data showing 93.3% long-term survival of 19-28z/mIL-12 CAR T cell-treated mice compared to 6.67% survival in 19-28z CAR T cell-treated mice ($p<0.0001$, n=15 from two independent experiments). 19-28z CAR T cell-treated mice have significantly enhanced survival compared to Pz1 ($p<0.0001$) and Pz1/mIL-12 ($p<0.0001$) CAR T cell-treated mice. Pz1/mIL-12 CAR T cell-treated mice do not have enhanced survival compared to Pz1 CAR T cell-treated mice ($p=0.6291$).

19-28z/hIL-12 CAR T cell therapy has reduced anti-tumor efficacy compared to 19-28z/mIL-12 CAR T cell therapy *in vivo*

While 19-28z/mIL-12 CAR T cell therapy improved anti-tumor efficacy compared to 19-28z CAR T cell treatment, it remains unclear whether the effect of IL-12 is entirely autocrine as murine IL-12 can have an effect on many cell types. In a

SCID-Beige mouse, the B and T cell compartments are compromised due to the Prkdc mutation, and the *beige* mutation impairs NK cell cytotoxic activity (Croy and Chapeau, 1990; Roder and Duwe, 1979). IL-12 has been demonstrated to enhance NK cell function and rescue NK cell activity in the context of STAT-1 deficiency, so it is possible that mIL-12 may rescue the impaired NK cell activity in SCID-Beige mice (Lee et al., 2000). Moreover, macrophages derived from *beige* mice are capable of macrophage cytotoxicity factor (MCF)-induced lysis and antibody-dependent cell-mediated lysis (ADCC) (Meerpohl et al., 1976; Roder and Duwe, 1979). Macrophages also express IL-12Rβ2 and IL-12 stimulation resulted in a positive feedback loop that amplifies IL-12 secretion (Grohmann et al., 2001).

To test whether the enhanced anti-tumor efficacy in 19-28z/mIL-12 CAR T cell therapy may in part be attributed to paracrine effect of IL-12 on NK cells or macrophages, we generated 19-28z/hIL-12 CAR by replacing the murine flexi-IL-12 region of 19-28z/mIL-12 with human flexi-IL-12 (Wagner et al., 2004). Since human IL-12 is not cross-reactive with mouse IL-12 receptor, treating a SCID-Beige mouse with 19-28z/hIL-12 should not affect host cells. We injected NALM-6 tumor cells into SCID-Beige mice followed by infusion with 19-28z, 19-28z/mIL-12, or 19-28z/hIL-12 CAR T cells. As noted previously, 19-28z/mIL-12-treated mice had significantly greater overall survival compared to 19-28z-treated mice ($p<0.0001$, $n=10$). The majority (90%) of 19-28z/mIL-12-treated mice were tumor-free until day 69, and overall 60% of all mice survived on day 150 (Figure 5.2A). The 19-28z/hIL-12-treated mice also had significantly greater survival compared to 19-28z-treated mice with 20% overall survival on day 150 ($p=0.0497$, $n=10$). Differential tumor growth can also be observed

as early as day 34 after tumor administration as determined by bioluminescent imaging (Figure 5.2B).

However, it is noteworthy that the overall survival of 19-28z/hIL-12 CAR T cell-treated mice was significantly lower compared to 19-28z/mIL-12 CAR T cell-treated mice ($p=0.0488$, $n=10$). This suggests that the enhanced efficacy of 19-28z/mIL-12 CAR T cells may in part be due to a paracrine effect of IL-12 on NK cells and/or macrophages. While NK cell activity is reduced in SCID-Beige mice, it is possible that IL-12 or other IL-12-induced cytokines can rescue this defect, resulting in enhanced NK cell-mediated anti-tumor activity. IL-12 may also induce macrophages towards an M1 phenotype, supporting enhanced anti-tumor activity.

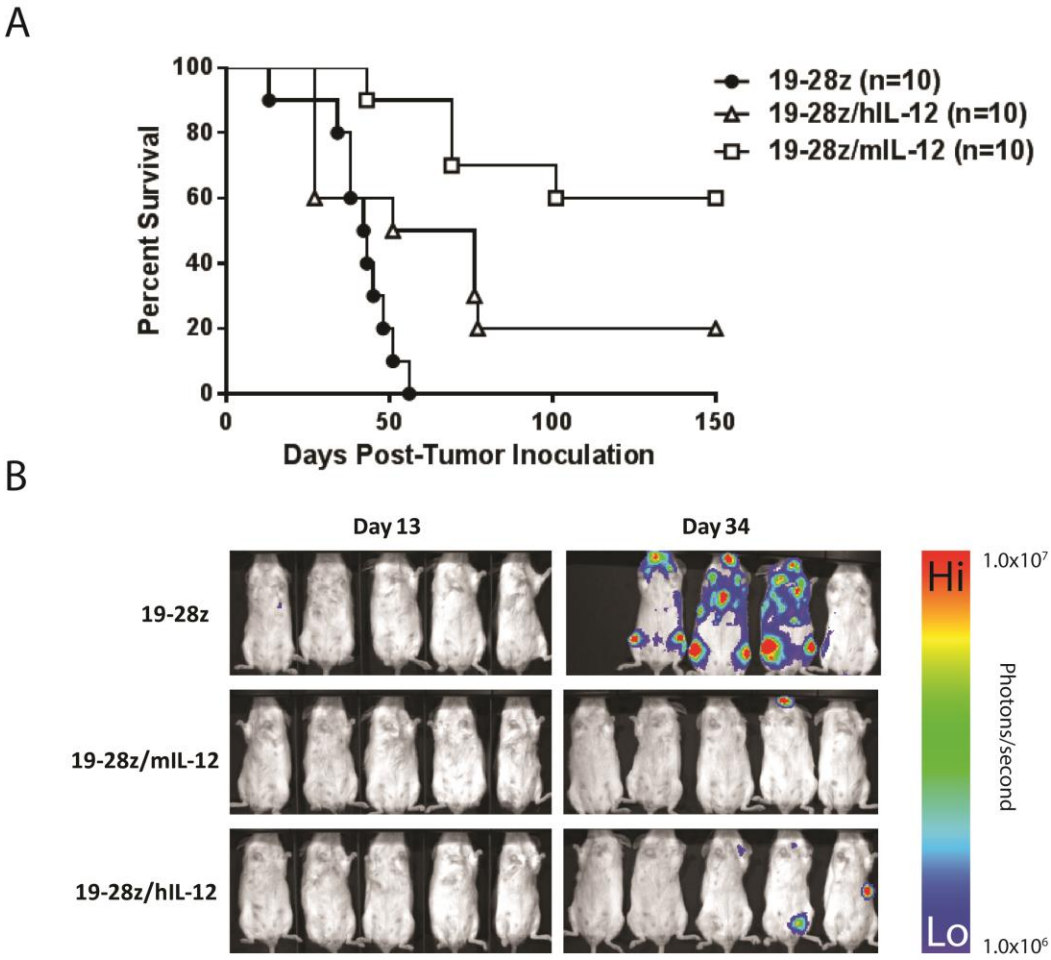

Figure 5.2 19-28z/hIL-12 CAR T cell therapy has reduced anti-tumor efficacy compared to 19-28z/mIL-12 CAR T cell therapy *in vivo*
(A) SCID-Beige mice were inoculated (i.v.) with $1x10^6$ NALM-6/GFPLuc tumor cells on day 0 and $1x10^7$ CAR T cells on day 6. Data showing 60% long-term survival of 19-28z/mIL-12 CAR T cell-treated mice compared to 20% survival in 19-28z/hIL-12 CAR T cell-treated mice (p=0.0488). 19-28z/mIL-12 ($p<0.0001$) and 19-28z/hIL-12 (p=0.0497) T cell-treated mice have enhanced survival compared to 19-28z T cell-treated mice in which none of the mice in the latter group survived. n=10 mice from two independent experiments. (B) Representative bioluminescent imaging (BLI) of tumor progression following i.v. administration of NALM-6 tumor on day 13 and day 34.

CAR T cell persistence *in vivo*

To further explain the mechanism of enhanced 19-28z/mIL-12 CAR T cell-mediated *in vivo* anti-tumor efficacy, we assessed persistence of CAR T cells in peripheral blood of 19-28z, 19-28z/mIL-12, and 19-28z/hIL-12 CAR T cell-treated mice at seven and 14 days after CAR T cell infusion. No significant difference in percentage of $CD3^+CAR^+$ T cells can be observed between 19-28z, 19-28z/mIL-12, and 19-28z/hIL-12 CAR T cell-treated mice at seven days post-T cell administration as determined by flow cytometry on peripheral blood samples from treated mice (Figure 5.3A). Furthermore, $CD3^+CAR^+$ T cells were no longer detectable at 14 days post-T cell administration (Figure 5.3B).

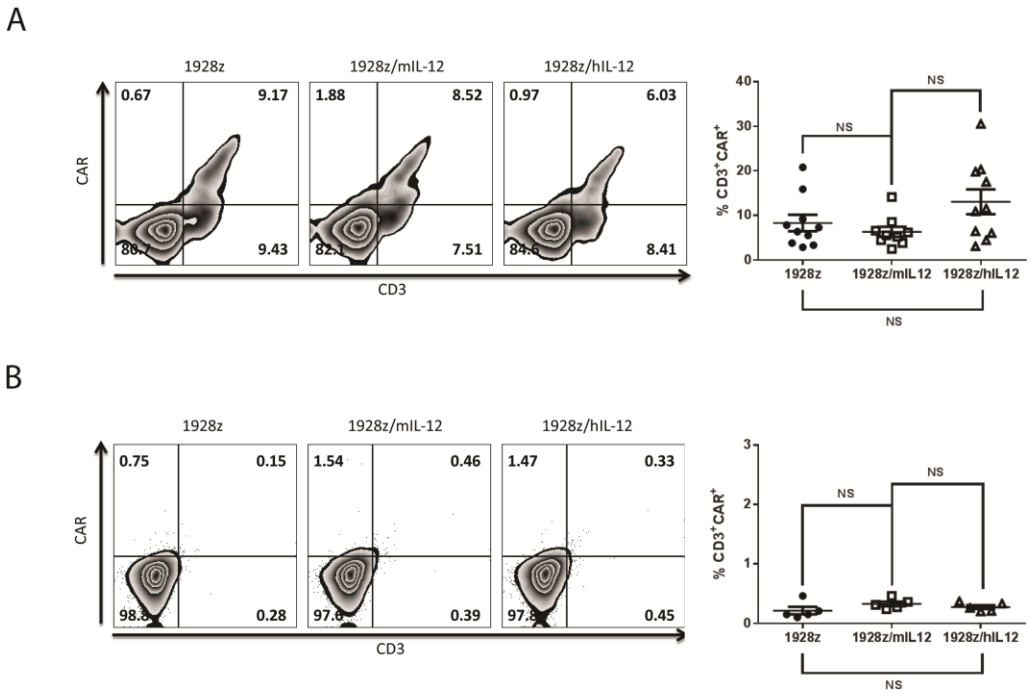

Figure 5.3 CAR T cell persistence in NALM-6 tumor-bearing mice
SCID-Beige mice were inoculated (i.v.) with $1x10^6$ NALM-6 tumor cells on day 0 and $1x10^7$ CAR T cells on day 14. At seven days (A) and 14 days (B) after CAR T cell infusion, peripheral blood was evaluated for CAR and CD3 expression by flow cytometry. (A) Representative data (left panel) and summary (right panel) of frequency of $CD3^+CAR^+$ T cells at seven days post-T cell injection of ten mice from two independent experiments. (B) Representative data (left panel) and summary (right panel) of frequency of $CD3^+CAR^+$ T cells at 14 days post-T cell injection of five mice from two independent experiments. NS, p=not significant.

CHAPTER 6: IL-12-SECRETING CAR T CELLS RESIST TREG SUPPRESSION

Exogenous IL-12 induces 19-28z CAR T cell resistance to nTreg suppression

Having established the effect of mIL-12 in enhancing anti-tumor efficacy of CAR T cells both *in vitro* and *in vivo*, we next considered whether mIL-12 can mediate nTreg resistance in human CAR T cells. We hypothesized that mIL-12-stimulated CAR T cells may resist nTreg suppression, considering the enhanced proliferation and cytotoxic potential of mIL-12-stimulated CAR T cells. IL-2 and IL-15 have also been demonstrated to enhance anti-tumor efficacy of T cell therapy (Nastala et al., 1994; Rosenberg et al., 1986). We therefore tested whether these cytokines may improve resistance to nTreg suppression.

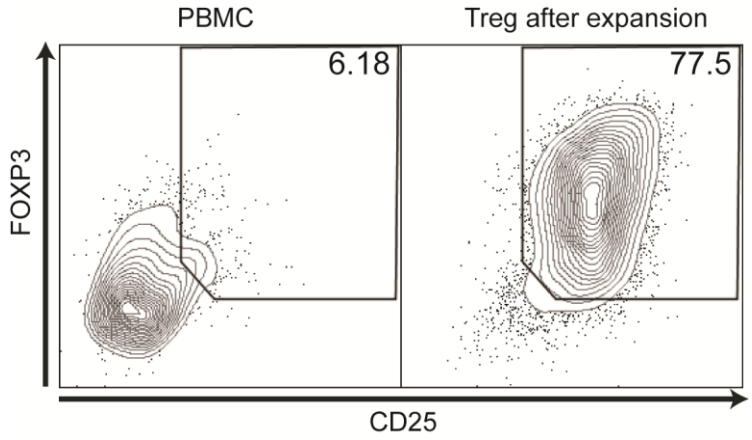

Figure 6.1 nTreg FOXP3 expression after expansion
$CD4^{+}CD25^{hi}$ nTregs were isolated from peripheral blood by magnetic bead separation and expanded with anti-CD3/CD28 beads in the presence of IL-2 and rapamycin. Data showing FOXP3 and CD25 expression after three weeks of expansion as assessed by flow cytometry.

We isolated nTregs from PBMCs, and generated a viable population of nTregs that retained high FOXP3 expression after three weeks of expansion (Figure 6.1). We then co-cultured the expanded nTregs with activated CAR T cells in the presence of IL-2, murine IL-12, and IL-15. nTregs suppressed the proliferation of 19-28z CAR T cells in response to anti-CD3/CD28 bead stimulation, resulting in a significant reduction in CAR T cell proliferation (36.55%) compared to 19-28z CAR T cells cultured without nTregs (60.67%) after 72 hours of anti-CD3/CD28 bead stimulation ($p=0.0152$, $n=6$; Figure 6.2). The addition of exogenous mIL-12 significantly protected the capacity of 19-28z CAR T cells to proliferate in response to stimulation in the presence of nTregs (57.45%) ($p=0.0260$, $n=6$). In contrast, culturing 19-28z CAR T cells in the presence of IL-2 ($p=0.0931$, $n=6$) or IL-15 ($p=0.3052$, $n=6$) had no significant effect on nTreg resistance. Furthermore, we did not observe any nTreg resistance in 19-28z CAR T cells cultured in the presence of IFN-γ ($p=0.7619$, $n=3$), IL-7 ($p=0.1667$, $n=3$), IL-18 ($p=0.7143$), IL-21 ($p=0.2619$, $n=3$), and IL-23 ($p=0.4762$, $n=3$; Figure 6.3).

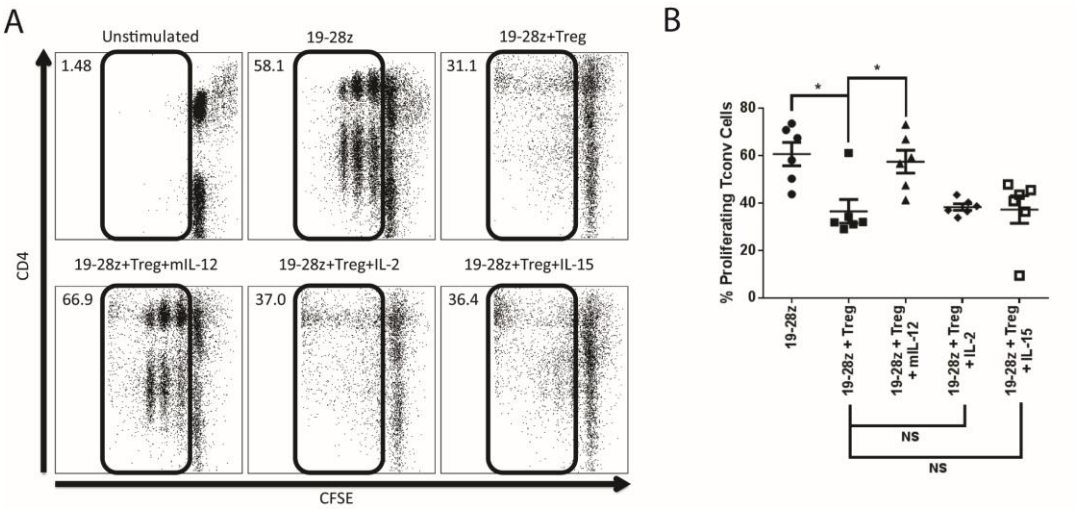

Figure 6.2 19-28z CAR T cells resist nTreg suppression of proliferation in the presence of mIL-12

$5x10^5$ CFSE-labeled 19-28z CAR T cells were stimulated with anti-CD3/CD28 beads and co-cultured with $5x10^5$ nTregs in the presence of exogenous IL-2, IL-12, or IL-15 for 72 hours. (A) Representative data showing the percentage of proliferating T cells gated in the CFSE-diluted population. (B) Combined summary of six independent experiments. *$p<0.05$; NS, p=not significant.

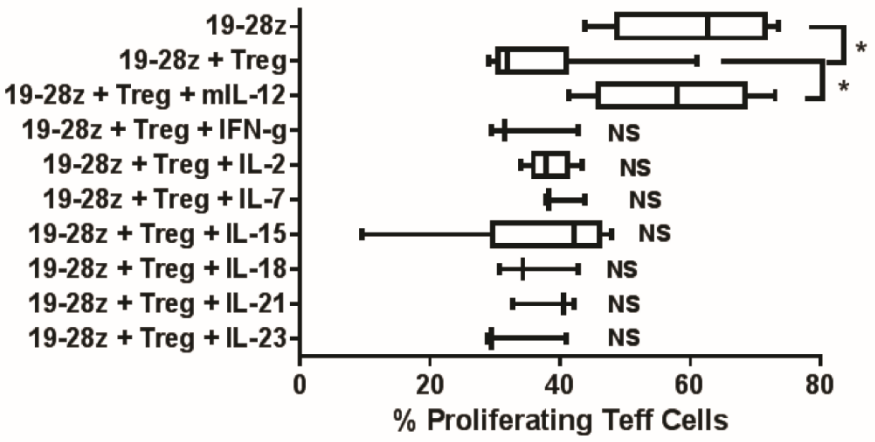

Figure 6.3 IFN-γ, IL-7, IL-18, IL-21, and IL-23 do not have an effect on the capacity of 19-28z CAR T cells to resist nTreg suppression
$5x10^5$ CFSE-labeled 19-28z CAR T cells were stimulated with anti-CD3/CD28 beads and co-cultured with $5x10^5$ nTregs in the presence of exogenous IL-12, IFN-γ, IL-2, IL-7, IL-15, IL-18, IL-21, and IL-23 for 72 hours. *$p<0.05$, n=3; NS, p=not significant relative to the "19-28z + nTreg" group.

We further tested whether mIL-12 can protect the cytolytic capacity of 19-28z CAR T cells in the presence of nTregs. We co-cultured 19-28z CAR T cells in the presence or absence of nTregs for 24 hours at a 1:1 ratio followed by a 24 hour co-culture with CD19-expressing Raji tumor cells. In contrast to Pz1 CAR T cells (which targets PSMA), 19-28z CAR T cells fully eradicated $CD19^+$ Raji tumor cells in the absence of nTregs (Figure 6.4). While nTregs inhibit the ability of the 19-28z CAR T cells to kill Raji tumor cells, 19-28z CAR T cells can overcome this suppression in the presence of exogenous mIL-12. This was not observed in the presence of exogenous IL-2 or IL-15 (Figure 6.4).

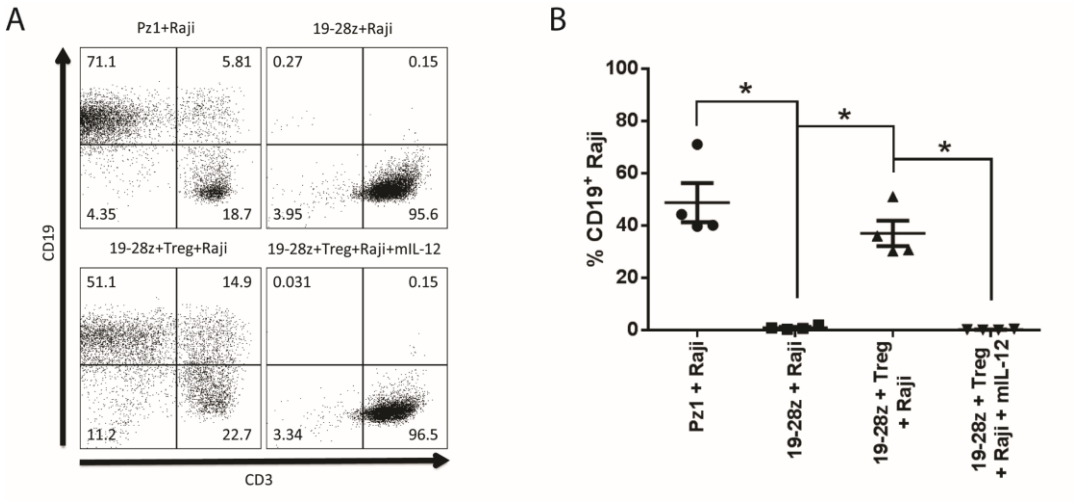

Figure 6.4 19-28z CAR T cells resist nTreg suppression of cytotoxic activity in the presence of mIL-12

CAR T cells were co-cultured with nTregs at a 1:1 nTreg:CAR ratio for 24 hours. Raji cells were then added in the presence or absence of mIL-12 for an additional 24 hours. (A) Representative data showing the percentage of $CD19^+$ tumor cells and $CD3^+$ T cells after co-culture as determined by flow cytometry after anti-CD19 and anti-CD3 antibody staining. (B) Summary of four independent experiments. *$p<0.05$, n=4.

19-28z/mIL-12 CAR T cells resist nTreg suppression *in vitro*

To further assess whether 19-28z/mIL-12 CAR T cells similarly have the capacity to resist nTreg suppression, we performed a CFSE proliferation assay with T cells transduced to express 19-28z or 19-28z/mIL-12. We co-cultured 19-28z or 19-28z/mIL-12 CAR T cells in the presence of nTregs and evaluated their proliferative capacity by measuring CFSE dilution after 72 hours of anti-CD3/CD28 bead stimulation. As we have demonstrated earlier, 19-28z CAR T cells had significantly reduced proliferation in the presence of nTreg (10.2%) compared to 19-28z CAR T cells cultured in the absence of nTregs (57.6%) after anti-CD3/CD28 bead stimulation ($p=0.05$, n=3)

(Figure 6.5A-B). In contrast, 19-28z/mIL-12 CAR T cells retained proliferative capacity in the presence of nTregs (64.9%) after anti-CD3/CD28 bead stimulation ($p=0.05$; Figure 6.5A-B).

We next investigated whether 19-28z/mIL-12 CAR T cells are also refractory to nTreg-mediated suppression of tumor lysis. We co-cultured 19-28z or 19-28z/mIL-12 CAR T cells in the presence nTregs for 24 hours at a 1:1 ratio followed by a 24 hour co-culture with CD19-expressing Raji tumor cells. We evaluated cytotoxic activity of CAR T cells based on the percentage of tumor cells that remained as assessed by flow cytometry. In contrast to 19-28z CAR T cells which had a reduced capacity to kill Raji tumor cells in the presence of nTregs, cytotoxic function of 19-28z/mIL-12 CAR T cells was maintained in the presence of nTregs (Figure 6.5C-D).

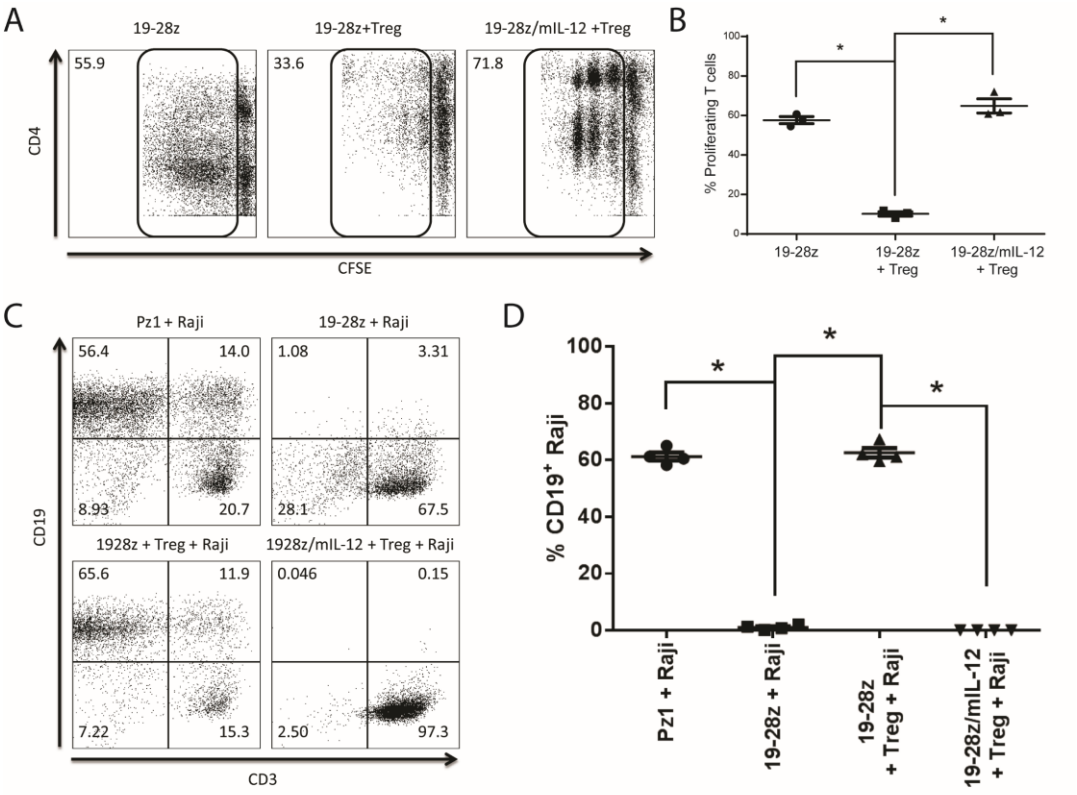

Figure 6.5 19-28z/mIL-12 CAR T cells resist nTreg suppression *in vitro*
(A-B) 19-28z/mIL-12 CAR T cells resist nTreg suppression of proliferation. CAR T cell proliferation after three days of anti-CD3/CD28 bead stimulation and nTreg co-culture. Data showing the percentage of proliferating T cells gated in the CFSE-diluted population (*p=0.05, n=3). (C-D) 19-28z/mIL-12 CAR T cells resist nTreg-mediated suppression of cytotoxicity. CAR T cells were co-cultured with nTregs at a 1:1 nTreg:CAR ratio for 24 hours. Raji cells were then added and the cells were analyzed by flow cytometry 24 hours later (*p<0.05, n=4).

19-28z/mIL-12 CAR T cells resist nTreg suppression *in vivo* and promote long-term survival of tumor-bearing mice

We have previously established an *in vivo* nTreg xenotransplant mouse model in which we showed that 19z1-transduced nTregs can traffic to the site of Raji tumor and markedly inhibit effector 19-28z CAR T cells (Teff) from eradicating the tumor.

We demonstrated that 19-28z CAR T cells lose its capacity to eradicate Raji tumors at a nTreg:Teff ratio of as low as 1:8 in a xenotransplant SCID-Beige mouse model (Lee et al., 2011). Here, we demonstrate that 19-28z/mIL-12 CAR T cell-treated mice had enhanced survival compared to control mice treated with 19-28z CAR T cells. The 19-28z/mIL-12 CAR T cell-treated mice had a long-term survival of 75%, even at a high nTreg:Teff ratio of 1:1 ($p<0.0001$, n=16; Figure 6.6).

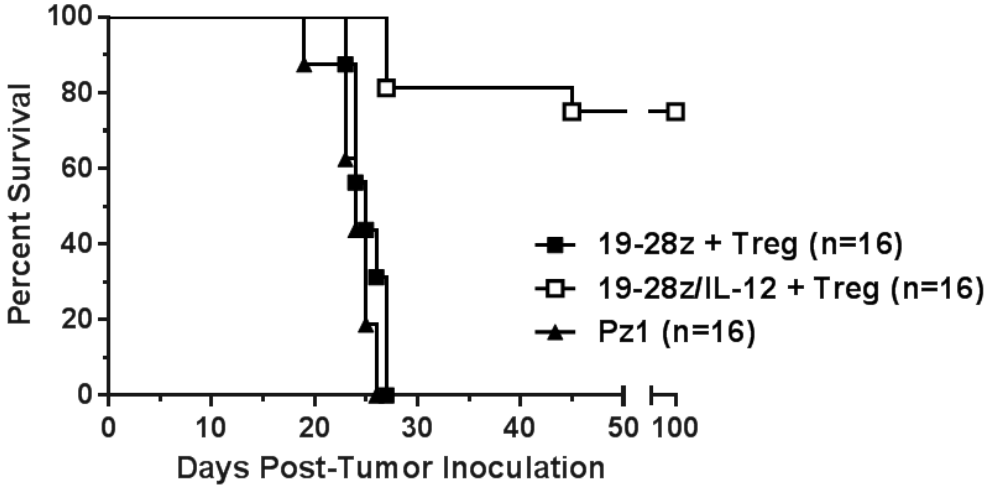

Figure 6.6 19-28z/mIL-12 CAR T cell therapy has enhanced anti-tumor efficacy compared to 19-28z CAR T cell therapy under nTreg-suppressing conditions *in vivo*

SCID-Beige mice were inoculated (i.v.) with $5x10^5$ Raji tumor cells on day 0, $1x10^7$ 19z1 CAR nTregs on day 5, and $1x10^7$ CAR T cells on day 6. 75% long-term survival of 19-28z/mIL-12 CAR T cell-treated mice compared to 0% survival of 19-28z CAR T cell-treated mice ($p<0.0001$, n=16 from three independent experiments). No significant difference between mouse treated with 19-28z CAR T cell compared to Pz1 CAR T cell-treated mice ($p=0.0618$).

CHAPTER 7: EFFECT OF IL-12 AND IFN-γ ON TREGS AND TUMOR CELLS

Having demonstrated the effect of IL-12 on the ability of CAR T cells to resist nTreg suppression both *in vitro* and *in vivo*, we next initiated studies investigating the underlying mechanisms of IL-12-mediated nTreg resistance. In our SCID-Beige nTreg xenotransplant model, there are three cell types that may potentially respond to IL-12 secretion in the tumor microenvironment (Figure 7.1): (1) CAR T cells may respond to IL-12 in an autocrine fashion allowing resistance to anti-proliferative signals. CAR T cells may also indirectly respond to IFN-γ, a major cytokine induced by IL-12, which can further enhance CAR T cell function. (2) nTregs may respond to IL-12 directly or indirectly through IFN-γ that dampens its suppressive capacity. (3) Raji tumor cells may respond to IL-12 or other IL-12-induced cytokines that may weaken the capacity of Raji tumor cells to survive in the tumor microenvironment.

IL-12-mediated nTreg resistance requires constant IL-12 stimulation

We first examined the contribution of IL-12 on CAR T cells. As we have demonstrated, IL-12 enhances the capacity of CAR T cells to proliferate and resist nTreg-mediated suppression. However, it is unclear if a single dose of IL-12 will induce permanent changes in the CAR T cells rendering them resistant to nTregs without further IL-12 stimulation. To address this question, we pre-incubated effector T cells (Teff) cells in the presence of mIL-12 for 24 hours then tested whether the Teff cells can still resist nTreg suppression after washing out the mIL-12 in the media. In contrast to Teff cells that were cultured in the presence of mIL-12, Teff cells pre-incubated with

mIL-12 (12Teff) cells no longer resist nTreg suppression after loss of mIL-12 stimulation (Figure 7.2). Similarly, Teff cells pre-incubated with IFN-γ (γTeff) do not have the capacity to resist nTreg suppression (Figure 7.2). These results are consistent with required persistent IL-12 stimulation of Teff cells as pre-incubation of Teff cells with IL-12 failed to result in retained resistance to nTregs.

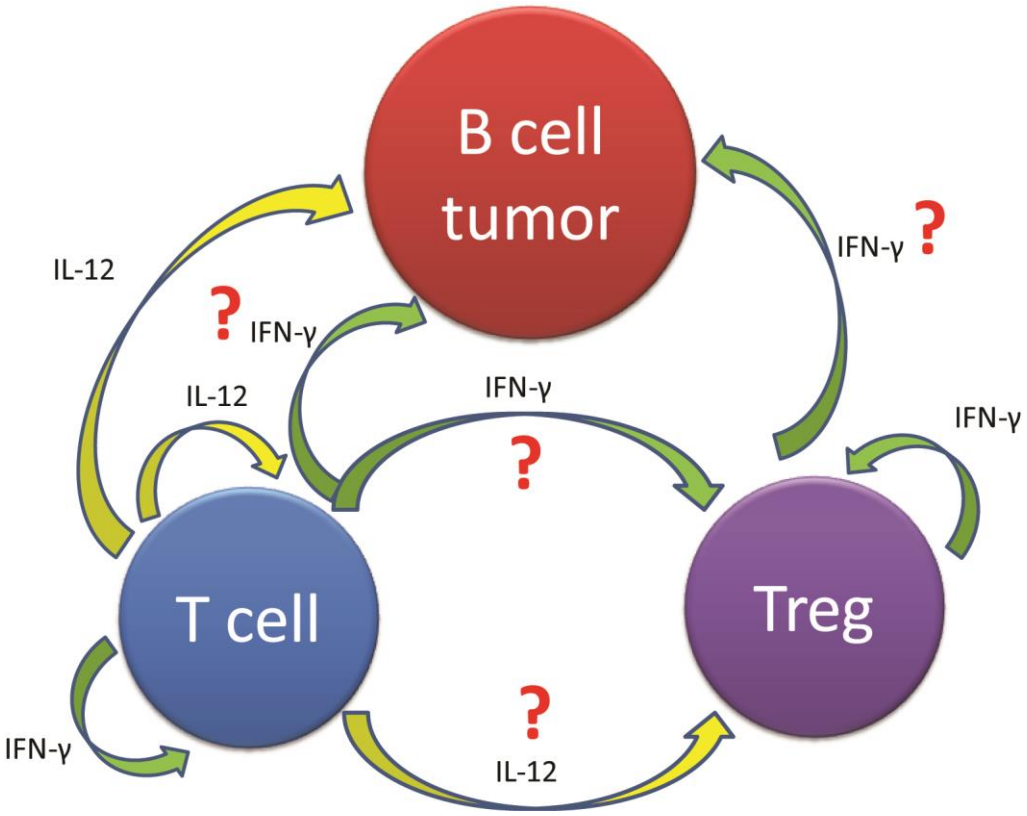

Figure 7.1 Mechanism of IL-12-mediated nTreg resistance
Schematic diagram depicting the three major cell types, CAR T cells, nTregs, and B cell tumor, that may potentially respond to IL-12 secretion in the tumor microenvironment. Hypothetically, IL-12 secreted by 19-28z/mIL-12 CAR T cells may have a direct effect on all three cell types or indirect effect through IFN-γ or other cytokines induced by IL-12

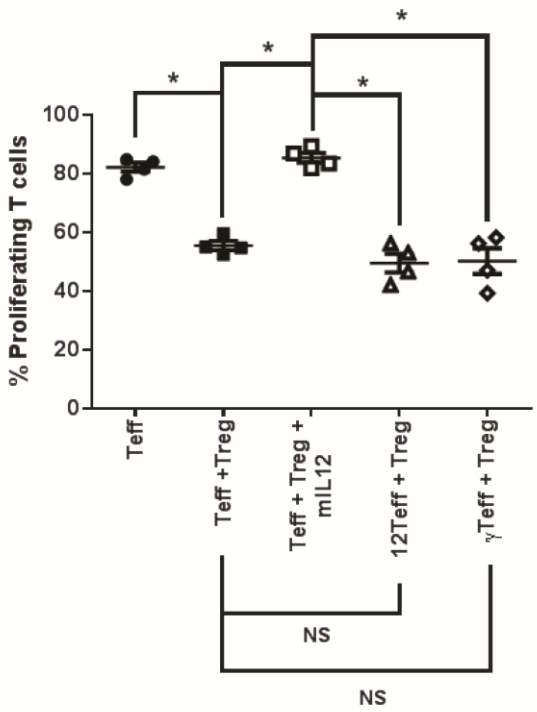

Figure 7.2 IL-12-mediated nTreg resistance requires constant IL-12 stimulation
CFSE-labeled Teff cells were pre-incubated with mIL-12 (12Teff) or IFN-γ (γTeff) for 24 hours. After thorough washing, $5x10^5$ of the pre-incubated Teff cells were co-cultured with $5x10^5$ nTregs for 72 hours after anti-CD3/CD28 bead stimulation. Control CFSE-labeled Teff cells were co-cultured with nTregs in the presence or absence of mIL-12. *$p<0.05$; NS, p=not significant.

IL-12-mediated nTreg resistance is not due to its effect on nTreg cells

To assess whether IL-12 secretion by CAR T cells may impact the function of nTregs, we first determined whether nTregs express the IL-12 and IFN-γ receptors to directly respond to these cytokines. We verified that a substantial percentage of nTregs expressed IL-12Rβ2 after 48 hours of activation, indicating that nTreg function may be influenced by IL-12 secretion (Figure 7.3A). Furthermore, a substantial percentage of

nTregs also expressed IFN-γR1, indicating that nTreg function may also be influenced by IFN-γ (Figure 7.3B).

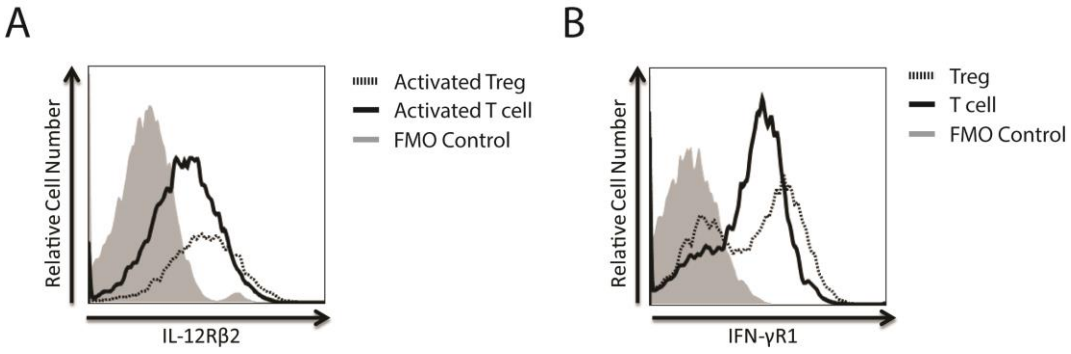

Figure 7.3 nTregs express IL-12Rβ2 and IFN-γR1
(A) IL-12Rβ2 expression on nTregs and T cells that were stimulated with anti-CD3/CD28 beads for 48 hours as determined by flow cytometry following staining with an antibody specific for IL-12Rβ2. (B) IFN-γR1 expression in resting T and nTreg cells as determined by flow cytometry following staining with an antibody specific for IFN-γR1.

Since nTregs express both IL-12 and IFN-γ receptors, we reasoned that one possible explanation for the reduced nTreg suppressive activity may be a direct impact of IL-12 or IFN-γ on Treg function. To test this hypothesis, we pre-cultured nTreg cells in the presence or absence of mIL-12 for 24 hours, washed out the mIL-12 in the media, then assessed whether the nTregs retain suppressive function. In a 72 hour CFSE anti-CD3/CD28 bead stimulated proliferation assay, we observed that nTregs no longer suppress Teff cells in the presence of mIL-12 as expected. However, nTregs that were pre-incubated with mIL-12 (12Treg) retained suppressive capacity (Figure 7.4). Similarly, nTregs that were pre-incubated with IFN-γ (γTreg) also retained suppressive

capacity (Figure 7.4). This suggests that the effect of IL-12 on nTreg resistance is not likely due to a direct impact of IL-12 or IFN-γ on the suppressive function of nTregs.

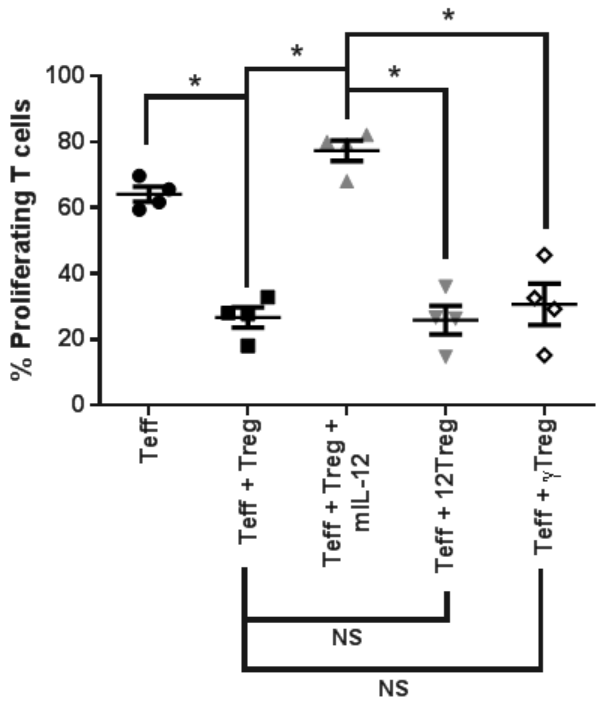

Figure 7.4 Effect of IL-12 and IFN-γ on nTreg suppression
nTreg cells were pre-incubated with mIL-12 (12Treg) or IFN-γ (γTreg) for 24 hours. After thorough washing, $5x10^5$ of the pre-incubated nTreg cells were co-cultured with $5x10^5$ CFSE-labeled Teff cells for 72 hours after anti-CD3/CD28 bead stimulation. Control CFSE-labeled Teff cells were co-cultured with nTregs in the presence or absence of mIL-12. *$p<0.05$; NS, p=not significant.

While IL-12 and IFN-γ do not appear to have a direct impact on the suppressive capacity of nTregs, we reasoned that IL-12-mediated nTreg resistance may be due to the impact of IL-12 or IFN-γ on the loss of the intrinsic proliferative capacity of nTregs. To determine whether IL-12 and IFN-γ have any effect on nTreg proliferation, we

cultured CFSE-labeled nTregs in the presence of IL-12 or IFN-γ for 72 hours after anti-CD3/CD28 stimulation. We demonstrated that IL-12 does not reduce nTreg proliferation but actually induces a significant increase in nTreg proliferation (Figure 7.5). Furthermore, we did not observe any difference in proliferation between nTregs that were stimulated in the presence or absence of IFN-γ.

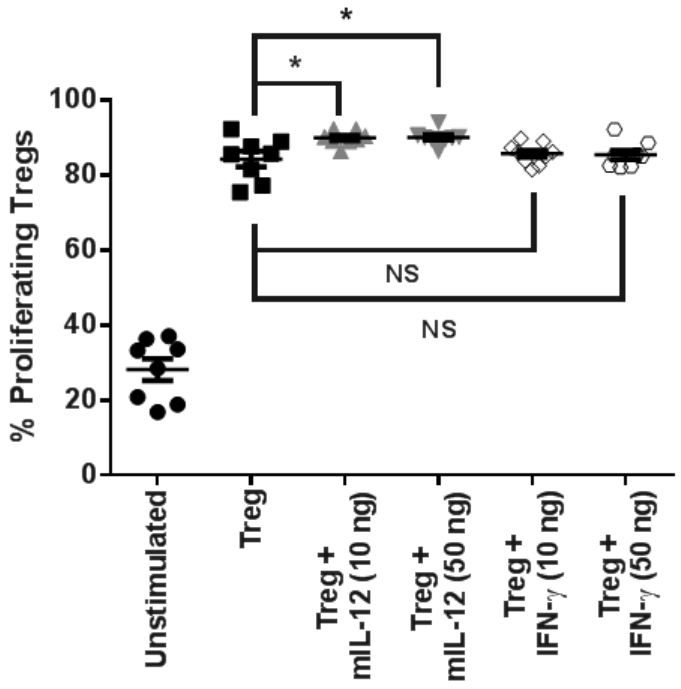

Figure 7.5 Effect of IL-12 and IFN-γ on nTreg proliferation
CFSE-labeled nTregs were stimulated with anti-CD3/CD28 beads in the presence or absence of IL-12 or IFN-γ for 72 hours. Percentage of proliferating nTregs was determined by evaluating the CFSE-diluted T cells among the $FOXP3^+$ cell population. *$p<0.05$; NS, p=not significant.

IL-12 has no intrinsic effect on Raji tumor cells

To assess whether IL-12 may directly affect tumor viability, we next investigated the effect of IL-12 or IFN-γ on Raji tumor cells. We first determined whether Raji tumor cells express IL-12 or IFN-γ receptors to directly respond to these cytokines. No expression of IL-12Rβ2 was observed in Raji tumor cells (Figure 7.6A). However, Raji tumor cells do express IFN-γR1 (Figure 7.6B).

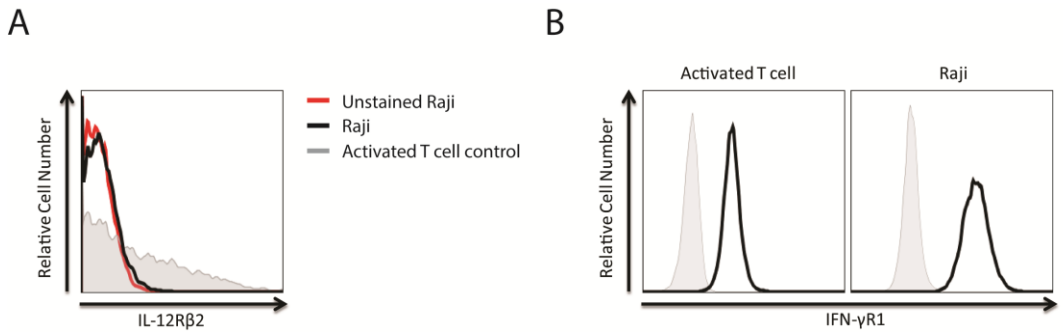

Figure 7.6 Expression of IL-12Rβ2 and IFN-γR1 in Raji tumor cells
Raji cells and activated T cells were assessed for the expression of (A) IL-12Rβ2 and (B) IFN-γR1 by flow cytometry.

Since Raji cells may respond to IFN-γ, we tested whether IFN-γ can induce IL-12Rβ2 expression in Raji cells. To determine the effect of IFN-γ on IL-12Rβ2 expression in Raji cells, we cultured Raji cells at three different concentrations of IFN-γ. However, we do not observe any differential expression of IL-12Rβ2 compared to activated T cell control (Figure 7.7A). While IFN-γ may not have an effect on IL-12Rβ2, it is possible that IFN-γ may have a direct impact on the survival and proliferation of Raji cells. However, we did not observe any effect of IFN-γ on the survival of Raji tumor cells as assessed by the expression of BCL-2 in Raji cells after IFN-γ stimulation

(Figure 7.7B). Stimulating Raji cells with IFN-γ also had no significant effect on the ability of Raji cells to proliferate (Figure 7.7C).

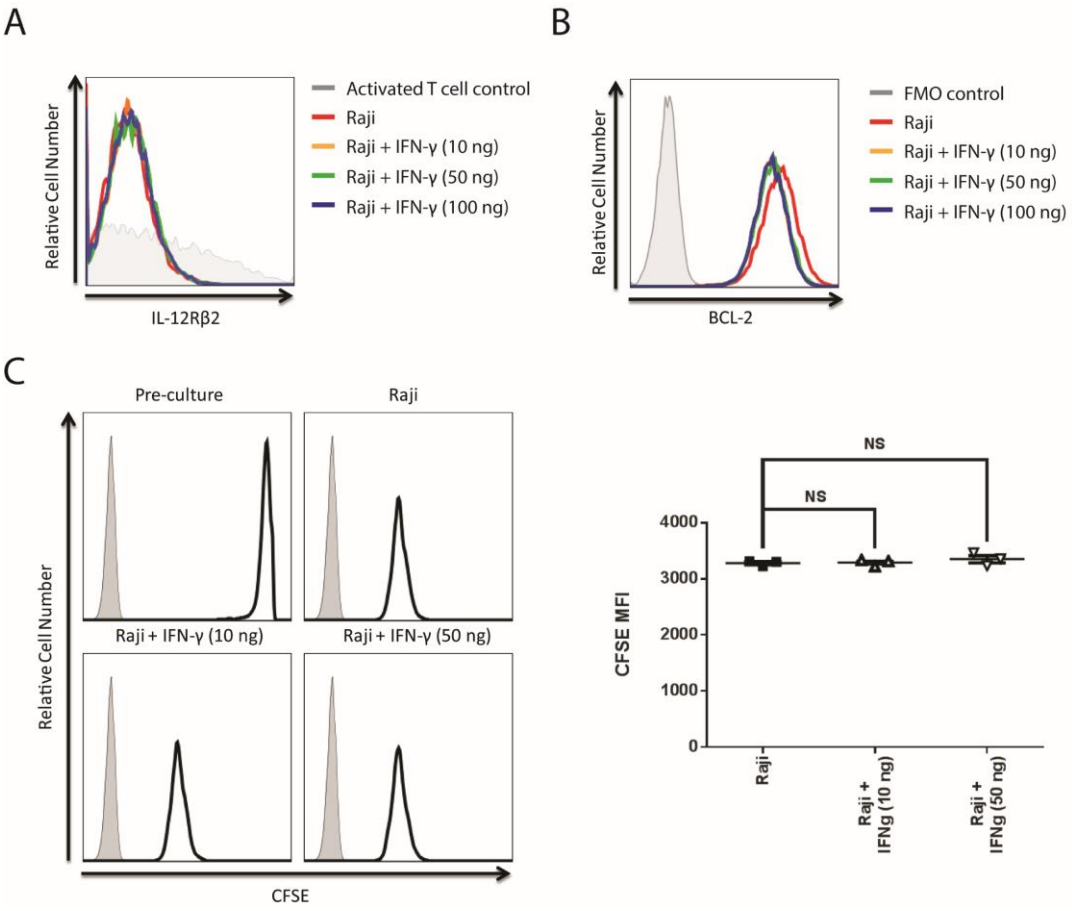

Figure 7.7 IFN-γ has no effect on Raji tumor cells
Raji cells were cultured in IFN-γ at the indicated concentrations for 24 hours and assessed for (A) IL-12Rβ2 and (B) BCL-2 expression by flow cytometry. (B) Raji cells were labeled with CFSE and cultured in IFN-γ at the indicated concentrations for 72 hours. Percentage of proliferating Raji cells was determined by evaluating CFSE-diluted Raji cells. Data represent three independent experiments. NS, p=not significant.

Since the 19-28z/mIL-12 CAR T cells have the potential to express many other cytokines besides IFN-γ upon antigen stimulation, it is possible that any of these other

cytokines may have an effect on Raji cells. To rule out this possibility, we cultured Raji cells in the supernatant of CAR T cells that have been previously activated with AAPCs and assessed IL-12Rβ2 expression. Whether the Raji cells were stimulated with 19-28z or 19-28z/mIL-12 CAR T cell supernatant, we did not observe any differential IL-12Rβ2 expression as compared to unstimulated Raji cells (Figure 7.8A). Stimulating Raji cells with the supernatant of 19-28z/mIL-12 or 4H11-28z/mIL-12 CAR T cells also have no observable effect on BCL-2 expression as compared to Raji cells stimulated with 19-28z or 4H11-28z CAR T cells, respectively (Figure 7.8B). Furthermore, co-culturing Raji cells with 4H11-28z and 4H11-28z/mIL-12 CAR T cells have no differential effect on the capacity of Raji cells to proliferate (Figure 7.8C). We conclude that IL-12-mediated nTreg resistance is not likely due to the impact of IL-12 on Raji tumor cells.

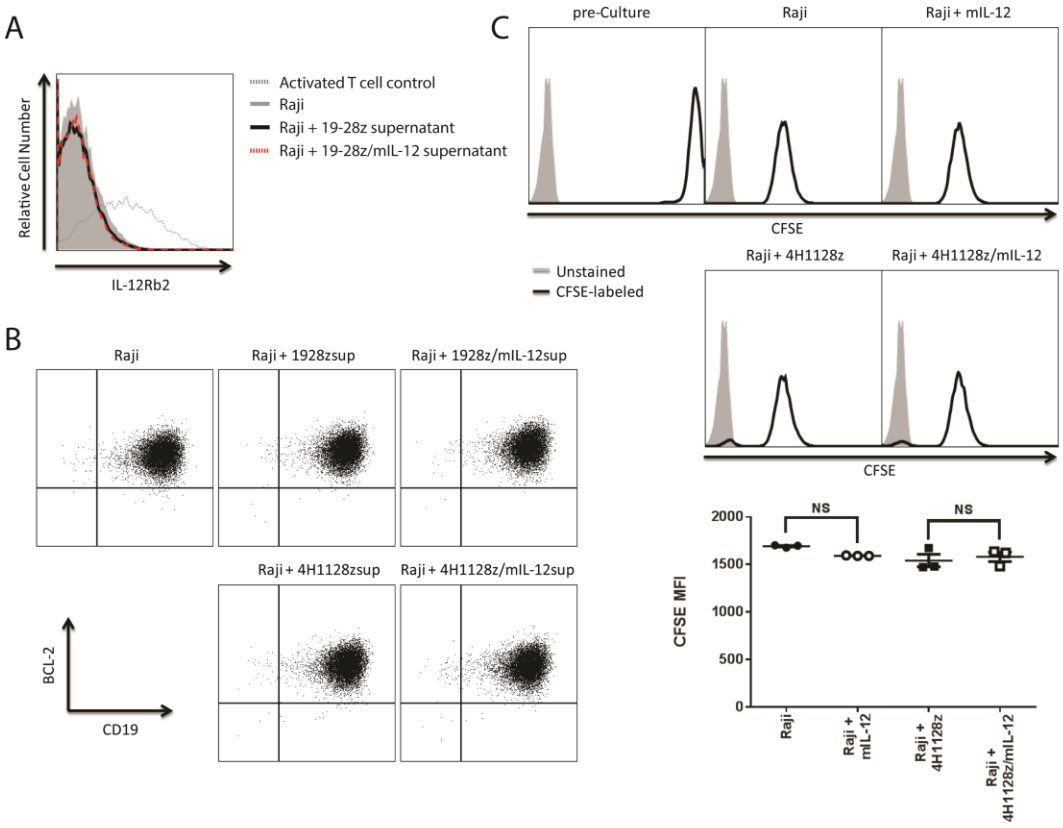

Figure 7.8 Cytokines secreted by 19-28/mIL-12 CAR T cells have no effect on Raji tumor cells

(A-B) $2x10^6$ CAR T cells were stimulated with 3T3-AAPC (3T3-CD19/CD80 or 3T3-MUC16/CD80) for 24 hours. The supernatant of T cell culture was used to culture Raji cells for an additional 24 hours. (A) IL-12Rβ2 and (B) BCL-2 expression were assessed by flow cytometry. Data represent three independent experiments. (C) $1x10^6$ CFSE-labeled Raji cells were co-cultured with $1x10^6$ activated 4H1128z or 4H1228z/mIL-12 CAR T cells for 72 hours. Control Raji cells were cultured in the presence or absence of mIL-12. Data represent three independent experiments. NS, p=not significant.

CHAPTER 8: DISCUSSION

The discipline of adoptive cell therapy (ACT), a form of cancer immunotherapy, started nearly half a century ago when it was first discovered that lymphocyte infiltration resulted in spontaneous cancer regression in several types of cancer (Rosenberg et al., 1972). As it became clear that these tumor-infiltrating lymphocytes (TILs) were responsible for cancer regression, cancer therapies that harness the anti-tumor faculty of immune cells have been developed over the years. While considerable progress has been made in recent years with the advent of tumor-specific TCR and CAR T cell engineering, successful application of cancer immunotherapy remains a challenge for most cancers (Duong et al., 2015).

Several important obstacles need to be addressed in order for an immunotherapy to successfully treat cancer. First, it is important to select a tumor-associated antigen (TAA) that is specific to the cancer but absent in normal cells to avoid on-target off-tumor toxicity. Engineered T cells that express TCR or CAR that recognize the TAA must be able to efficiently traffic to the tumor. Once at the site of the tumor, tumor-targeted T cells must receive both signal 1 (TCR) and signal 2 (co-stimulation) to be fully activated and overcome anergy. Upon activation, tumor-targeted T cells must be able to expand adequately and persist long enough to eradicate cancer. Moreover, the T cells must survive and retain function in a tumor microenvironment. This includes the presence of immunosuppressive Tregs, TAMs, and MDSCs as well as expression of inhibitory factors such as PD-L1, TGF-β and IL-10.

Our studies described a novel cancer immunotherapy that combines the clinical advantages of CAR T cells with the anti-tumor effect of localized IL-12 secretion. Such a therapy addresses many of the challenges of cancer immunotherapy. We demonstrated that IL-12 enhances proliferation, improves effector function, and confers T cells to resist Treg suppression in the context of cancer.

Tumor Associated Antigen

In our studies, 19-28z/mIL-12 CAR T cells specifically recognize the CD19 antigen expressed on normal B cells as well as B cell tumors. CD19 antigen is a nearly ideal TAA as it is expressed in virtually all forms of B cell leukemia and lymphoma. While CD19 is also expressed in most stages of B cell development, it is absent in pluripotent stem cells in the bone marrow. CD19-targeted CAR T cell therapy may lead to B cell aplasia as a result of on-target off-tumor toxicity. However, the toxic effect of B cell aplasia can be countered by therapeutic immunoglobulin infusion and restored by *de novo* B cell differentiation from pluripotent stem cells in the bone marrow after therapy.

T Cell Activation and Proliferation

Classical T cell activation requires two signals – TCR signaling upon antigen recognition (signal 1) and a co-stimulatory signal provided by activated antigen presenting cells (signal 2). Absence of the latter signal induces activated T cells into a non-responsive state known as anergy (Smith-Garvin et al., 2009). Activated T cells eventually succumb to activation-induced cell death (AICD) through Fas ligation-induced caspase activation. On the other hand, co-stimulatory signaling such as CD28

binding to CD80 or CD86 have been shown to upregulate survival signals BCL-XL and FLIP while downregulating Fas ligand (FasL) (Budd, 2001). In addition to the two primary signals, Mescher and colleagues demonstrated that IL-12 can serve as a signal 3, which is required for activation of naïve T cells but not antigen-experienced memory T cells (Schmidt and Mescher, 2002). Indeed, first generation CAR T cells that received only signal 1 lack the capacity to proliferate and persist compared to second generation CAR T cells that received both signals 1 and 2 (Savoldo et al., 2011). Second generation CAR T cells may demonstrate superior anti-tumor efficacy against tumor compared to first generation CAR T cells, but repeated expansion of such T cells results in eventual exhaustion in the absence of survival factors such as IL-15 (Brentjens et al., 2003).

In our studies, we provided signal 3 by means of exogenous IL-12 or constitutive secretion of IL-12 by the CAR T cells. In both cases, we demonstrated that IL-12 enhances the proliferative capacity of CAR T cells (Figure 3.1, 4.5). Moreover, 19-28z/mIL-12 CAR T cells retain this proliferative capacity after repeated antigen exposure (Figure 4.7). Others have also reported that IL-12 induces proliferation and long term survival of T cells *in vitro* (Bertagnolli et al., 1992; Salgado et al., 2002). Our data are consistent with Mescher's studies, demonstrating that IL-12 is required for clonal expansion and enhanced cytotoxic activity of T cells (Curtsinger et al., 2003).

IL-12-Secreting CAR T Cells

Despite enhanced anti-tumor efficacy of systemic IL-12 as signal 3 in preclinical studies, early clinical trials using systemic high dose IL-12 infusion resulted in severe toxicity in which patients developed flu-like symptoms, rapid leukopenia, and liver

dysfunction that in some cases was fatal (Car et al., 1999; Leonard et al., 1997). To further explore the anti-tumor effects of IL-12, it is therefore imperative to develop a delivery system that allows IL-12 to be selectively administered directly within the tumor microenvironment. Wagner and colleagues first explored this possibility by demonstrating that "flexi-IL-12"-transduced EBV-specific CTLs produce IL-12 only in the presence of antigenic stimulation (Wagner et al., 2004).

Several recent reports further established that it is possible to deliver IL-12 to the site of the tumor and induce tumor regression by adoptively transferring IL-12-transduced tumor-targeted T cells. We reported in a hCD19 transgenic C57BL/6 EL4 mouse tumor model that mIL-12-secreting CD19-targeted CAR T cells eradicated tumor in the absence of prior lymphodepletion with no evidence of systemic toxicity (Pegram et al., 2012). Restifo and colleagues demonstrated in a murine Pmel-1 melanoma model that tumor-targeted T cells modified to express IL-12 can enhance anti-tumor activity of tumor-targeted T cells (Kerkar et al., 2010). Similarly, treatment with human IL-12-secreting CAR T cells targeted to the angiogenic VEGF receptor successfully eradicated five different types of syngeneic tumors, including melanoma, sarcoma, and colorectal adenocarcinoma (Chinnasamy et al., 2012). Chmielewski and colleagues also demonstrated that treatment with CAR T cells engineered to release inducible IL-12 upon CAR engagement resulted in tumor eradication that is dependent on macrophage activation (Chmielewski et al., 2011).

Central Memory Phenotype and T Cell Persistence

Resting T cells exist as naïve (T_N), central memory (T_{CM}), and effector memory (T_{EM}) T cell populations. Whereas T_N cells are $CD45RA^+CD45RO^-$, T_{EM} and T_{CM} are $CD45RA^-CD45RO^+$. T_N cells and T_{CM} cells are $CCR7^+CD62L^+$ while T_{EM} cells are $CCR7^-CD62L^-$ (Mueller et al., 2013). It has been demonstrated using mouse OT-I cells that exogenous IL-12 priming induces the survival of $CD8^+CD62L^{hi}$ T_{CM}-like cells, which results in enhanced anti-tumor activity following adoptive cell transfer (Díaz-Montero et al., 2008). A T_{CM}-like phenotype may be clinically relevant as Riddell and colleagues have demonstrated in macaque monkeys that adoptively transferred CMV-specific T cells derived from $CD8^+CD62L^+$ T_{CM}-like cells have long term persistence when compared to transfer of Teff cells (Berger et al., 2008). More recently, Riddell and colleagues also demonstrated the synergistic improvement in survival of Raji tumor-bearing NOD scid gamma (NSG) mice treated with CD19-targeted CAR T cells derived from a combination of CD4 and CD8 T_{CM} subsets (Sommermeyer et al., 2015). As a result of these studies, clinical grade CD19-targeted T cells derived from $CD8^+CD62L^+$ T cells are being tested in clinical trials of B cell malignancy (Terakura et al., 2012; Wang et al., 2011b).

Consistent with these studies, we found that a higher proportion of the 19-28z/mIL-12 CAR T cells display a $CCR7^+CD62L^+$ T_{CM}-like phenotype (Figure 4.6). Among the $CCR7^+CD62L^+$ population, some of the T cells display CD45RO expression while others display CD45RA expression (data not shown). As all the CAR T cells were antigen-experienced, the $CD45RA^+CCR7^+CD62L^+$ population in our data are not

typical T_N cells. Restifo and colleagues have recently described a new class of memory T cell subset known as T stem cell memory (T_{SCM}) characterized by $CD45RO^-$ $CD45RA^+CCR7^+CD62L^+CD95^+IL\text{-}2R\beta^+$ which has the highest proliferative capacity and anti-tumor efficacy among memory T cells (Gattinoni et al., 2011). It is possible that the T_N-like cells seen in 19-28z/mIL-12 CAR T cells are actually T_{SCM} cells, though this requires further investigation. Additionally, future studies on the effect of IL-12 on T_{SCM} differentiation will need to be formally tested.

To further test whether this enhanced $CCR7^+CD62L^+$ T_{CM}-like phenotype seen in 19-28z/mIL-12 CAR T cells may result in enhanced *in vivo* persistence, we infused 19-28z and 19-28z/mIL-12 CAR T cells into tumor-bearing mice and assessed CAR T cell persistence at seven and 14 days post-infusion. In our study, we did not observe significant difference in persistence of 19-28z/IL-12 CAR T cells in our pre-clinical xenotransplant model as compared to 19-28z CAR T cells (Figure 5.3). However, in an ovarian xenotransplant solid tumor model, Koneru and colleagues observed enhanced persistence in 4H11-28z/IL-12 CAR T cells as compared to 4H11-28z CAR T cells (Koneru et al., 2015). In this latter model, the route of CAR T cell injection was intraperitoneal (i.p.) rather than systemic (i.v.) as in our studies, and relative rate of tumor growth, as well as the number of injected CAR T cells ($2.5x10^6$ vs. $1x10^7$) all differ. These differences may all contribute to the persistence of CAR T cells *in vivo*. It remains to be seen whether IL-12-secreting CAR T cells may exhibit enhanced persistence in the clinical setting.

Cytotoxic Potential

The primary role of CAR T cells in cancer immunotherapy is to initiate its cytotoxic function on the tumor cells. Our studies demonstrated that IL-12 enhances the production of granzyme B and perforin, two molecules essential for the cytotoxic activity of CTLs (Figure 3.2 and Figure 4.9A-B). Furthermore, co-culture of 19-28z/mIL-12 CAR T cells with NALM-6 tumor cells resulted in reduced tumor cell survival compared to co-culture of 19-28z CAR T cells with NALM-6 tumor (Figure 4.9D). Consistent with 19-28z/mIL-12 human CAR T cells, we have also demonstrated that IL-12-secreting murine CAR T cells (19mz/mIL-12) have elevated granzyme B and perforin production and have enhanced cytotoxic activity (Pegram et al., 2012). These data are consistent with the established function of IL-12 in enhancing cytotoxic activity of T lymphocytes (Aste-Amezaga et al., 1994). In addition to enhancing cytotoxic activity of T lymphocytes, IL-12 is also a potent inducer of NK cell killing (Kobayashi et al., 1989). Therefore, IL-12 secretion in 19-28z/IL-12 CAR T cell therapy has the potential to enhance killing of tumor cells by means of both autocrine T cell and paracrine NK cell stimulations.

IL-12 and Treg Resistance

In the tumor microenvironment, it has been well-established the important role of Tregs in suppressing anti-tumor efficacy of immunotherapy (Roychoudhuri et al., 2015). Therefore, generating tumor-targeted T cells capable of resisting Treg suppression is an important step towards improving cancer immunotherapy. Many institutions have previously performed mouse studies suggesting the possible role of IL-

12 in resisting Treg-mediated inhibition of Teff cells (Cao et al., 2009; Curran et al., 2012; Feng et al., 2011; King and Segal, 2005; Koch et al., 2012; Zhao et al., 2012).

However, an *in vivo* human T cell model demonstrating IL-12-mediated resistance to Treg suppression in the context of cancer has not yet to date been reported. Moreover, the underlying mechanism of IL-12-mediated Treg resistance remains controversial. For example, King and Segal proposed that IL-12-mediated $CD4^+CD25^-$ T cell resistance to Treg inhibition is IFN-γ independent as mouse Treg-Teff co-cultures with IFN-γ failed to restore Teff cell function (King and Segal, 2005). Zhao and colleagues further demonstrated that while IL-12 induces increased IFN-γ expression in mouse Tregs, IFN-γ^+ and IFN-γ^- Tregs inhibit Teff cells equally well (Zhao et al., 2012). Feng and colleagues similarly demonstrated that $FOXP3^+$ mouse Tregs can convert into IFN-γ^+FOXP3^+ Tregs in the presence of TGF-β and IL-12 that retain suppressive capacity (Feng et al., 2011). In contrast, Cao and colleagues showed evidence that IFN-γ is at least in part responsible for IL-12-mediated reduction of mouse Treg cell survival as IL-12 failed to inhibit IFN-$\gamma R1^{-/-}$ Treg cell expansion *in vitro* (Cao et al., 2009). In a syngeneic hCD19 transgenic C57BL/6 EL4 mouse tumor model, Pegram and colleagues also suggested that IFN-γ is likely responsible for Treg resistance as no mice survived following treatment with CD19-targeted IL-12-secreting CAR T cells derived from IFN-γ deficient mice (Pegram et al., 2012). Treg resistance may also be attributed to the capacity of IL-12-secreting cells to resist the immunosuppressive cytokine TGF-β, a signature Treg cytokine. As Wagner and colleagues demonstrated, "Flexi-IL-12 CTLs" can resist TGF-β-mediated inhibition of proliferation and cytotoxic activity *in vitro* (Wagner et al., 2004).

Our studies demonstrated for the first time the ability of human CAR T cells to resist Tregs in the context of IL-12. Both exogenous IL-12 stimulation and IL-12-secreting CAR T cells have the ability to resist Treg suppression of proliferation and cytotoxic activity of CAR T cells (Figure 6.2, 6.4, 6.5). Furthermore, this ability to resist Treg suppression as seen in 19-28z/mIL-12 CAR T cells is also observed in the context of an *in vivo* Raji tumor xenotransplant mouse model. Compared to mice treated with 19-28z CAR T cells, 19-28z/mIL-12 CAR T cell treatment resulted in improved survival of mice previously injected with tumor-targeted immunosuppressive Tregs (Figure 6.7).

Our data suggest that the ability of 19-28z/mIL-12 CAR T cells to resist Treg suppression is not due to a paracrine effect of IL-12 on Raji tumor cells. We demonstrated that Raji cells do not respond to IL-12 stimulation (Figure 7.6), and other cytokines secreted by activated 19-28z/mIL-12 CAR T cells, including IFN-γ, have no effect on the proliferation and expression of the survival signal BCL-2 in Raji cells (Figure 7.7, 7.8).

Therefore, IL-12-mediated resistance of Treg suppression is likely due to the effect of IL-12 on CAR T cells and/or Tregs. We demonstrated that both CAR T cells and Tregs express the receptors required to respond to IL-12 and IFN-γ stimulation (Figure 7.3). Stimulating Tregs with IL-12 resulted in a slight increase in the proliferation of Tregs, while stimulating Tregs with IFN-γ had no effect on Treg proliferation (Figure 7.5). To distinguish whether the effect of IL-12 was due to its effect on CAR T cells or Tregs, we pre-incubated CAR T cells or Tregs with IL-12 before testing the suppressive capacity of the Tregs. However, once the IL-12 was removed,

the Tregs retained its capacity to suppress CAR T cell proliferation in both cases (Figure 7.2 and 7.4). These data suggest that pre-incubating CAR T cells or Tregs with IL-12 does not permanently change the phenotype of CAR T cells or Tregs, resulting in resistance to suppression. In other words, IL-12 stimulation must be present at the time of CAR T cell and Treg co-culture to resist Treg suppression.

To definitively distinguish whether IL-12-mediated resistance to suppression is due to the effect of IL-12 on T cells or Tregs, the following experiments will be performed in the future. We will perform the 72 hour IL-12-stimulated CFSE proliferation assay in the context of IL-12Rβ2 knockout (KO) in the Teff cells, the Tregs, or both cell types. Genetic knockout of IL-12Rβ2 may be achieved by using the CRISPR/Cas9 system prior to Teff-Treg co-culture (La Russa and Qi, 2015). In the context of normal Teff cells cultured with Treg IL-12Rβ2 KO cells, if the Teff cells can still proliferate in the context of IL-12 stimulation, then IL-12-mediated resistance to Treg suppression is likely due to an autocrine effect of IL-12 on Teff cells. This conclusion should be confirmed with the demonstration that Teff IL-12Rβ2 KO cells fail to resist suppression by normal Tregs in the context of IL-12 stimulation. We should also expect Treg IL-12Rβ2 KO cells to retain suppression of Teff IL-12Rβ2 KO cells in the context of IL-12 stimulation as neither Teff cells nor Tregs would be able to respond to IL-12 stimulation. As IL-12 is a potent inducer of IFN-γ, we can further test the contribution of IFN-γ by performing the same proliferation experiments in the context of IFN-γR1 KO in the Teff cells, the Tregs, or both cell types.

In addition to the *in vitro* CFSE proliferation experiments, we can further distinguish the effect of IL-12 on the adoptively transferred CAR T cells and the endogenous cells in a syngeneic tumor mouse model. Pegram and colleagues have demonstrated in a hCD19 transgenic C57BL/6 EL4 mouse tumor model that treatment with mIL-12-secreting CD19-targeted CAR T cells derived from C57BL/6 IL-12Rβ2 deficient background resulted in reduced anti-tumor efficacy compared to treatment with cells derived from normal C57BL/6 mice (Pegram et al., 2012). This suggests that an autocrine effect of IL-12 on the transferred CAR T cells is necessary to resist Treg suppression. We can further test whether the effect of IL-12 on Tregs is necessary for resistance to Treg suppression *in vivo* by performing a syngeneic tumor model using FOXP3-Cre mice with the IL-12Rβ2 gene locus flanked by LoxP sites. This results in specific deletion of IL-12Rβ2 gene in the $FOXP3^+$ Treg population. We can then test whether IL-12-secreting CAR T cells retain anti-tumor efficacy in this context. If true, then a paracrine effect of IL-12 on endogenous Tregs is not required for resistance to Treg suppression *in vivo*. Once we have eliminated the possibility that resistance to suppression requires a paracrine effect of IL-12 on Tregs, we can further study the mechanism of IL-12-mediated resistance to Treg suppression by performing gene arrays of the Teff cells that have been co-cultured with Tregs and IL-12 compared to the absence of IL-12 stimulation. Specific genes can then be identified and further tested by genetic knockout experiments.

While our studies do not suggest any direct effect of IL-12 on Treg function and survival, IL-12 may have an indirect effect on the recruitment and function of Tregs, resulting in an environment that favors an anti-tumor response. Our *in vitro* cytokine

secretion study demonstrated that IL-12 may decrease the production of certain cytokines associated with Treg recruitment and function. Among the cytokines we observed, TARC/CCL17, MDC/CCL22, I-309/CCL1, IL-9, and IL-16 secretion were reduced in the IL-12-stimulated group compared to non-IL-12-stimulated controls (Appendix 1). Sakaguchi and colleagues have described the preferential recruitment of $CCR4^+$ Tregs in melanoma tissue (Sugiyama et al., 2013). Since CCR4 responds to both TARC and MDC, it can be hypothesized that reduction in the production of TARC and MDC may result in decreased Treg recruitment in a syngeneic immunocompromised system. $CCR8^+FOXP3^+$ cells represent about 60% of all $FOXP3^+CD4^+$ memory T cells (Soler et al., 2006). Since I-309 recruits $CCR8^+$ T cells, reduced I-309 secretion may also reduce Treg recruitment. IL-16 has also been demonstrated to both recruit existing Tregs as well as induce *de novo* $FOXP3^+$ T cells (McFadden et al., 2007). Furthermore, IL-9 also enhances the suppressive function of $FOXP3^+$ Treg cells (Elyaman et al., 2009). Our xenotransplant immunocompromised mouse studies do not adequately account for the possible role of IL-12 in Treg recruitment. Future studies in a syngeneic tumor transplant model will need to be tested to formally address the role of these individual cytokines in IL-12-mediated Treg resistance *in vivo*.

Effect of IL-12 on Myeloid Cells

The benefit of using IL-12-secreting CAR T cell therapy likely extends beyond overcoming Treg-mediated inhibition. MDSCs, TAMs, and dendritic cells play a significant immunosuppressive role in many solid tumors (Gajewski et al., 2006). Restifo and colleagues proposed that IL-12 may enhance anti-tumor efficacy by

reprogramming MDSCs, macrophages, and dendritic cells to favor the support of CAR T cells (Kerkar et al., 2011). Evidence also suggests the importance of IL-12-mediated upregulation of Fas in myeloid cells with respect to tumor regression (Kerkar et al., 2013).

In our studies, we observed that 19-28z/mIL-12 CAR T cells are characterized by a Th1/Tc1-type immune response, producing high levels of the Th1 cytokine IFN-γ and low levels of the Th2 cytokines IL-5 and IL-13, and the Th17 cytokine IL-17 (Figure 3.3, 4.4). High levels of IL-12-induced IFN-γ are especially effective in reprogramming macrophages towards an M1 phenotype, shifting away from the immunosuppressive M2 phenotype associated with TAMs (Martinez and Gordon, 2014).

In Vivo Tumor Model

In a NALM-6 SCID-Beige model, we demonstrated that 19-28z/mIL-12 CAR T cells significantly improved survival of NALM-6 tumor-bearing mice when compared to 19-28z CAR T cell treatment. A single infusion of 19-28z/mIL-12 CAR T cells resulted in 93.3% survival of treated mice (Figure 5.1). In contrast, treatment with 19-28z CAR T cells resulted in only 6.67% long-term survival. This result is consistent with IL-12-secreting CAR therapy administration in a syngeneic immunocompromised system, where CD19 tumor-bearing mice treated with IL-12-secreting CAR T cells also resulted in improved survival compared to survival of mice treated with non-IL-12-secreting CAR T cells (Pegram et al., 2012). Furthermore, we demonstrated that the enhanced anti-tumor efficacy of 19-28z/mIL-12 CAR T cells is not entirely autocrine as 19-28z/hIL-12 CAR T cell treatment resulted in lower overall mouse survival (20%)

compared to 19-28z/mIL-12 CAR T cell treatment (60%) on day 150 after tumor injection (Figure 5.2). This suggests that IL-12 secretion by 19-28z CAR T cells may have a paracrine effect on residual NK cells and/or macrophages, mediating indirect tumor lysis that contributed to the observed enhanced mouse survival after CAR T cell treatment.

Until the development of 19-28z/mIL-12 CAR T cells, successful treatment of NALM-6 ALL tumor *in vivo* has been a challenge. Treating NALM-6 with multiple weekly doses of 19-28z CAR T cells resulted in merely 44% overall survival (Brentjens et al., 2007). Therefore, the significant improved survival as seen in treating NALM-6 tumor with 19-28z/mIL-12 CAR T cells is an important step towards translating IL-12-secreting CAR T cells to the clinic. As Pegram and colleagues demonstrated in a syngeneic EL4-tumor model, conditioning therapy as has been the current standard for CAR therapy may be eliminated when treating with CD19-targeted IL-12-secreting CAR therapy (Pegram et al., 2012).

Concluding Remarks

In this thesis, we present evidence that adoptively transferring tumor-targeted CAR T cells "armored" to secrete IL-12 may be a more effective approach to CAR T cell therapy for cancer. 19-28z/mIL-12 CAR T cells exhibit enhanced *in vivo* anti-tumor efficacy, enhanced proliferation, and resistance to Treg suppression. In contrast to toxicities reported in other pre-clinical studies of tumor targeted IL-12 secreting T cells, we did not observe any toxicity in 19-28z/mIL-12 CAR T cell-treated mice in this and previous studies (Kerkar et al., 2010; Pegram et al., 2012). This lack of associated

toxicity may in part be due to lower levels of IL-12 expression by our 19-28z/mIL-12 CAR T cells. In our retroviral construct, the flexi-IL-12 gene was placed behind an IRES element, which lowers the expression of IL-12 in these T cells (Kim et al., 2011). Nevertheless, we acknowledge the inherent risk associated with cytokine therapy and plan to include a suicide vector such as inducible caspase-9 or truncated EGFR to enhance safety (Straathof et al., 2005; Wang et al., 2011a).

Development of CAR T cell engineering for the treatment of cancer has come a long way from demonstrating the capacity of first generation CAR T cells to respond to antigen independent of HLA restriction to successful treatment of hematopoietic malignancies with second generation CAR T cells in clinical trials. We have learned the many obstacles associated with CAR T cell therapies, including the requirement to overcome the suppressive tumor microenvironment and the need to control cytokine release syndrome (CRS). The future of CAR T cell technology lies in overcoming these obstacles as we translate this therapy into the clinic. In this thesis, we have described one possible way to overcome these obstacles by introducing IL-12 in the CAR T cell environment. Our preclinical data demonstrated enhanced *in vivo* anti-tumor efficacy of CD19-targeted CAR T cells "armored" to secrete IL-12 which can be attributed to T_{CM}-like phenotype, enhanced cytotoxicity, and resistance to Treg suppression. Our successful preclinical studies suggest its potential clinical application for the treatment of B-CLL as well as the treatment of solid tumors.

Successful translation of IL-12-secreting CAR T cell therapy in the clinic remains to be seen. Combining IL-12 secretion and CAR T cell engineering opens up a

new avenue for CAR T cell research. Should IL-12-secreting CAR T cell therapy fail to deliver efficacy in the clinic, CAR T cells "armored" with other more powerful immunomodulatory cytokines may be tested for cancer immunotherapy in the future. Perhaps, a combination of these cytokines may be essential for delivering optimal anti-tumor response. As it became clear that several inhibitory factors such as CTLA-4 and PD-1 play a critical role in suppressing T cells in cancer, applying CAR therapy in combination with antibodies that neutralize such inhibitory factors is another area of CAR T cell research. As the study of CAR therapy has been focused mainly on T cells thus far, therapeutic application of this technology to other immune cell types may be explored in the future. As we continue to develop novel CAR T cell therapies for cancer, it is our sincere hope that safe CAR T cell therapy will one day be the standard of care for the treatment of cancer.

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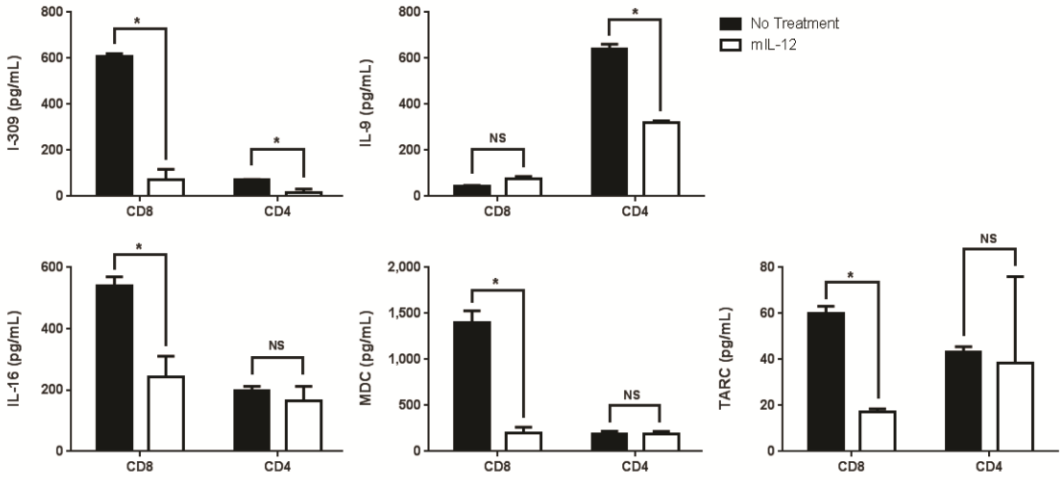

Appendix 1. Exogenous IL-12 reduces 19-28z CAR T cell secretion of cytokines that enhance Treg recruitment and function

19-28z CAR T cells were stimulated with anti-CD3/CD28 beads in the presence or absence of exogenous mIL-12 for 48 hours and the supernatant was assessed by luminex. Secretion of I-309, IL-9, IL-16, MDC, and TARC in CAR T cells after stimulation. *$p<0.05$, n=3; NS, p=not significant.